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# ASCORBIC ACID CONTENT OF GOAT'S MILK AND BLOOD: INFLUENCE OF ASCORBIC ACID INJECTION AND DIET [1]

MARTHA S. RICHMOND,[2] G. H. SATTERFIELD, C. D. GRINNELLS AND W. J. DANN

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THREE FIGURES

(Received for publication April 3, 1940)

Hunt and Winter ('22) found that 20 to 30 cc. of goat's milk daily protected guinea pigs from scurvy; their bio-assays indicated that goat's milk contained somewhat more vitamin C than cow's milk. Meyer and Nassau ('24) and Frank ('25) reported the contrary result, while Ruijter de Wildt and Brouwer ('30) found approximate equality in the vitamin C content of milks of the two species. Chakraborty ('35) found in five samples of goat's milk from 0.7 to 1.1 mg. of ascorbic acid per 100 gm. Rudra ('36) found 8.5 mg. per 100 cc. Cimmino ('38) found a mean value of 4.5 mg. per 100 cc. for four samples. These results are all based on bio-assays or on the titration of a small number of samples.

Since the commencement of our study an extensive report has been made by Gamble, Ellis and Besley ('39) on the composition of goats' milk. Vitamin C was determined by bio-assays during the years 1929–30–31, supplemented by chemical determinations at monthly intervals during the period June,

[1] Published with the approval of the Acting Director of the North Carolina Agricultural Experiment Station.

[2] Submitted in partial fulfillment of the requirements for the degree of Master of Science, North Carolina State College of the University of North Carolina. Aided by Chemical Foundation Scholarship.



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1936 to June, 1937. The handling of the milk for the later chemical tests was kept as nearly as possible the same as for the earlier bio-assays. The method of estimation was to precipitate the milk proteins with trichloracetic acid, filter, and titrate the filtrate with 2,6-dichlorophenol-indophenol. Individual samples contained from 0.5 to 2.5 mg. of ascorbic acid, with a mean of 1.3 mg., per 100 cc. of milk. Spring and summer milks appeared to have a slightly higher titre than fall and winter milks. It was suggested that the relative abundance of green forage was probably the most important factor determining the variations.

Sharp ('36) stated that the vitamin C content of fresh milk is not increased by the daily intrajugular injection of 4 gm. vitamin C in the goat.

Wendt ('38) reported that the ascorbic acid content of cow's milk had a relatively constant value of about 20 mg. per liter and that it was unaffected by the injection of vitamin C.

On the other hand, Rasmussen, Bogart and Maynard ('38) found temporary rises in ascorbic acid content of ewe's milk and of cow's milk within 14 hours after the intravenous injection of ascorbic acid. The increase amounted to approximately 50% of the initial values.

## EXPERIMENTAL

*Animals used.* Two groups of goats were used in this work, the first consisting of five Toggenburgs and the second of four assorted goats. The pertinent history of the Toggenburgs is given below:

| GOAT | BORN | KIDDED | REMARKS |
|---|---|---|---|
| 794 | 5/25/32 | 6/ 6/38 | |
| 906 | 2/ 9/33 | 5/ 2/38 | |
| 1000 | 2/22/33 | 5/12/38 | |
| 10 | 5/20/37 | 6/17/38 | Kid of no. 1000 |
| 11 | 5/20/37 | 7/ 9/38 | Kid of no. 1000 |

Until 11/10/38 these goats were kept in a pen with their kids and were not milked regularly. On 11/10/38 they were put into individual stalls and then milked twice daily. On 4/25/39 they were turned out to pasture. Their daily ration consisted

of good quality hay (alfalfa hay until 2/14/39 and then lespedeza hay) and a grain mixture of 4 parts white corn, 3 parts cottonseed meal, 2 parts wheat bran and 1 part oats. Occasionally the grain mixture was replaced by oats alone.

On three occasions they were given injections of ascorbic acid [3] as follows: 1 gm. each on 2/21/39, 1 gm. each on 4/17/39 and 2 gm. each on 5/17/39. Each dose was dissolved in reboiled distilled water and injected intraperitoneally about 11 A.M. Milk samples were collected just before the injections, about 4 hours later, twice the following day, and then once daily in the morning until the effect of the injection was no longer noticed. Blood and urine samples were collected at some of these milking-times.

The second group of goats was as follows:

| GOAT | KIDDED | BREED | ON C-FREE DIET |
|---|---|---|---|
| 3 | 3/14/39 | Angora | 5/20/39 to 7/ 1/39 |
| 8 | 3/19/39 | Half Toggenburg | 7/ 3/39 to 9/30/39 |
| M 1 | 2/18/38 | Half Toggenburg | 4/19/39 to 7/ 1/39 |
| M 2 | 3/10/39 | Half Toggenburg | 4/19/39 to 5/20/39 and 7/ 1/39 to 9/30/39 |

Except during periods on a vitamin C-free diet these goats were kept in a pen with their kids and fed hay only until 5/10/39. On this date they were turned out to pasture with the Toggenburgs and given the same hay and grain ration. On account of a snagged shoulder, M 2 was kept in an outside pen from 6/12/39 to 7/1/39 and fed hay only. When kept on the vitamin C-free diet the goats were penned in a small house having a concrete floor covered with sawdust. The periods spent in this pen are shown above; the only food given was the grain mixture.

## PROCEDURE

Blood and milk samples were taken from each goat at approximately weekly intervals except between 10/15/38 and 2/2/39 when milk samples were taken twice weekly. The blood was drawn from the jugular vein. Samples were taken in the

[3] Our thanks are due to Merck and Company for the gift of the ascorbic acid used in this study.

morning and kept in an electrical refrigerator in dark glass bottles until titrated; bottles for the blood contained 20 mg. sodium oxalate and 10 mg. potassium cyanide per 10 cc. of blood.

The method of titrating milk and colostrum samples was essentially that of King ('37). Five cubic centimeters of milk was added to 3 cc. of a solution containing 8% acetic acid and 4% metaphosphoric acid in a 40 cc. capsule vial. This was titrated against 2,6-dichlorophenol-indophenol, using a modified Neale-Forbes titration assembly and adding the dye solution until one drop produced a faint pink visible for 30 seconds. Each titration was completed within 3 minutes. The dye solutions were standardized in a manner similar to that described by Tripp, Satterfield and Holmes ('37) at such a concentration that about 0.3 to 0.4 cc. was required for each titration. On account of high viscosity, colostrum samples were usually treated with more than 3 cc. of the acid mixture.

Vitamin C in blood was determined by a method found to be the most reliable in a series of comparative studies by Satterfield, Perlzweig and Dann ('37). Four cubic centimeters of plasma, which must be free from hemoglobin pigments, was mixed with 6 cc. of reboiled distilled water and 10 cc. of 4% metaphosphoric acid and filtered. Five cubic centimeter portions of the filtrate were titrated as above, with a dye equivalent to approximately 0.02 mg. ascorbic acid per cubic centimeter.

Several hundred milk and blood samples were titrated, and the individual results plotted. The graphs for one of the Toggenburgs and each of the other goats are reproduced in the figures.

## RESULTS

The complete data showing variation in ascorbic acid content of the blood and milk under normal conditions and the effect of injecting ascorbic acid are given for only one of the five Toggenburg goats because they are typical of the group (fig. 1). The range and mean values of the ascorbic acid content of the milk and blood of each of the five goats are given in table 1.

The mean value of ascorbic acid content of all milk samples, excepting those obtained within 2 weeks after injection, was 1.24 mg. per 100 cc.; and about 90% of the samples fell within the range 0.5 to 2.0 mg. per 100 cc. These figures agree well with the values obtained by Gamble, Ellis and Besley quoted

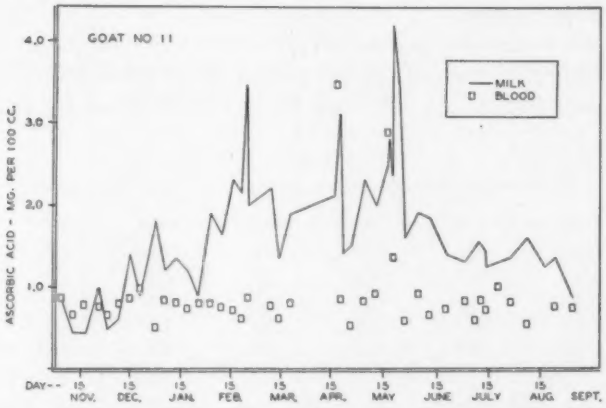


**Fig. 1** Vitamin C content of the milk and blood of a typical Toggenburg goat under normal conditions and after injection of 1 gm. ascorbic acid on 2/21 and 4/17 and 2 gm. on 5/17. Values obtained on 5/20 and 5/21 and given in table 3 are omitted because of the small scale of the figure.

TABLE 1

*Ascorbic acid content of Toggenburg milk and blood in milligrams per 100 cc.*

| | GOAT | NORMAL | | | AFTER INJECTION OF ASCORBIC ACID |
|---|---|---|---|---|---|
| | | Number of samples | Mean ascorbic acid | Standard deviation | Maximum ascorbic acid |
| Milk | 11 | 54 | 1.43 | 0.59 | 4.18 |
| | 10 | 53 | 1.36 | 0.74 | 4.02 |
| | 1000 | 53 | 1.30 | 0.58 | 4.53 |
| | 794 | 53 | 0.87 | 0.36 | 2.16 |
| | 906 | 53 | 1.16 | 0.73 | 3.03 |
| Blood | 11 | 30 | 0.77 | 0.10 | 3.46 |
| | 10 | 29 | 0.80 | 0.13 | 2.99 |
| | 1000 | 29 | 0.69 | 0.11 | 3.06 |
| | 794 | 29 | 0.56 | 0.11 | 2.47 |
| | 906 | 30 | 0.71 | 0.11 | 3.76 |

above. On the average they appear to be lower than the corresponding figures for cows' milk. Holmes, Tripp, Woelffer and Satterfield ('39) reported the mean values: for Guernsey cows 2.05, and for Holsteins 1.82 mg. per 100 cc. A survey of the literature shows that values for fresh cows' milk, protected from oxidation and irradiation, lie generally between 1.0 and 3.0 mg. per 100 cc.

Figure 1 shows the considerable variability of the ascorbic acid content of the milk under normal conditions, and the lack of close relationship between the concentrations in samples

TABLE 2

*Daily average milk production (pounds) of Toggenburg goats*

| GOAT | NOV. | DEC. | JAN. | FEB. | MAR. | APR. | MAY |
|---|---|---|---|---|---|---|---|
| 11 | 3.1 | 2.4 | 2.3 | 1.9 | 2.1 | 2.1 | 2.8 |
| 10 | 1.9 | 1.9 | 1.8 | 1.5 | 1.8 | 2.0 | 2.6 |
| 1000 | 3.4 | 3.4 | 3.6 | 2.8 | 3.3 | 2.8 | 3.4 |
| 794 | 2.1 | 1.8 | 1.6 | 1.4 | 1.6 | 1.6 | 2.0 |
| 906 | 2.1 | 1.8 | 1.4 | 1.1 | 1.3 | 0.9 | 0.8 |

of blood and milk taken at the same time. As an extreme example of the variation of concentration the following figures for no. 10 may be cited:

| DATE | 7/11 | | 7/12 | | 7/13 |
|---|---|---|---|---|---|
| | a.m. | p.m. | a.m. | p.m. | a.m. |
| Mg. ascorbic acid per 100 cc. milk | 1.34 | 2.22 | 0.98 | 1.60 | 0.71 |

The milk of four of the goats showed a small but definite increase over the period from November to February. A record was kept of the milk production of these goats for 7 months, and it was found that the yield varied widely between the different goats, and considerably for each goat from day to day. The variation was as great as that of the ascorbic acid concentration; sometimes in the same direction and at others in the opposite direction. The mean daily milk production for each month is given in table 2.

The effect of injection of ascorbic acid was first tested on 2/21/39 when each goat was given 1 gm. intraperitoneally. Blood samples were taken just before and 2 days after injec-

tion, so that the large temporary rise noticed after later injections was missed. The effect on the milk of the different animals was irregular at this test, as the next samples of two of the goats contained less ascorbic acid than samples taken just before injection. Examination of the urine showed a very large rise in ascorbic acid content within 4 hours after injection

TABLE 3

*Milligrams ascorbic acid per 100 cc. milk, blood and urine before and after injecting each goat with 2 gm. ascorbic acid*

| DATE | 5/10 | 5/17 a.m. | 5/17 p.m. | 5/18 a.m. | 5/18 p.m. | 5/19 | 5/20 | 5/21 | 5/22 | 5/26 a.m. | 5/26 p.m. | 6/3 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| MILK OF | | | | | | | | | | | | |
| 11 | 2.02 | 2.46[1] | 2.81 | 2.44 | 2.38 | *4.18*[2] | 2.98 | 2.66 | 3.28 | 1.62 | 1.48 | 1.96 |
| 10 | 1.91 | 2.69 | 2.82 | 2.27 | 2.17 | 3.79 | *4.02* | 2.75 | 3.40 | 1.06 | | 2.03 |
| 1000 | 1.39 | 1.90 | 1.90 | 1.71 | 1.85 | *3.28* | 3.06 | 2.07 | 2.68 | 1.45 | | 1.45 |
| 794 | 1.19 | 1.17 | 1.15 | 0.95 | 0.82 | 1.27 | 1.84 | 1.81 | *2.16* | 0.80 | | 0.81 |
| 906 | 2.61 | 0.39 | 0.80 | 0.57 | 0.38 | 0.67 | 1.17 | 1.49 | *2.38* | | 0.75 | 1.15 |
| BLOOD OF | | | | | | | | | | | | |
| 11 | 0.920 | | 2.87 | | | 1.28 | | | | 0.592 | | 0.905 |
| 10 | 0.766 | | 1.08 | | | 1.23 | | | | 0.712 | | 0.890 |
| 1000 | 0.743 | | 3.06 | | | 0.937 | | | | 0.442 | | 0.635 |
| 794 | 0.609 | | 2.47 | | | 0.962 | | | | 0.402 | | 0.548 |
| 906 | 0.502 | | 3.76 | | | 0.941 | | | | | 0.496 | 0.912 |
| URINE OF | | | | | | | | | | | | |
| 11 | | | 601 | 8.2 | | | | | | | | |
| 10 | | | 1.88 | 0.3 | | | | | | | | |
| 1000 | | | 112 | 5.8 | | | | | | | | |
| 794 | | | 136 | 0.5 | | | | | | | | |
| 906 | | | 241 | 3.0 | | | | | | | | |

[1] Injections were made immediately after these samples were collected.

[2] The italic figures were the maximum values observed after injection.

and a return to normal next morning. Following the second injection of each goat with 1 gm. of ascorbic acid on 4/17/39, it was found that after 4 hours the concentration in the blood was from two to five times as great as it was before injection, and that it returned to normal in about 2 days. In the milk the concentration rose between 50% and 100% for each of the goats.

The third injections, of 2 gm. ascorbic acid for each goat, were given on 5/17/39, and the complete data for the period immediately afterward are given in table 3. The next samples of blood drawn contained a greatly increased amount of ascorbic acid and the urine samples showed rapid excretion of the ascorbic acid, except for no. 10, where no significant change in

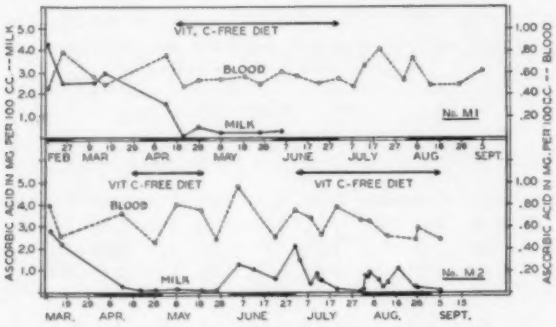


Fig. 2 Effect of diet on the vitamin C content of the milk and blood of two grade Toggenburg goats. Except for the vitamin C-free periods indicated, these goats received only hay until 5/10, after which they were turned on pasture and fed hay and grain.

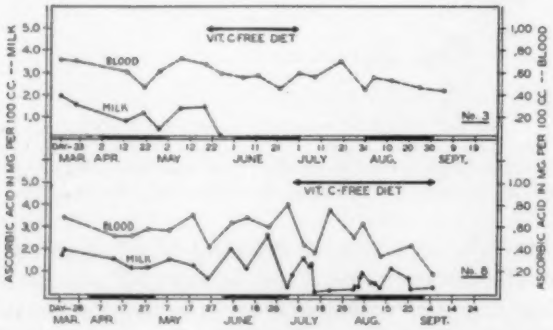


Fig. 3 Effect of diet on the vitamin C content of the milk and blood of an Angora goat (no. 3) and a grade Toggenburg (no. 8). Except for the vitamin C-free periods, these goats received only hay until 5/10, after which they were turned on pasture and fed hay and grain.

blood or urine occurred. It is feared that no. 10 was not injected intraperitoneally, as this goat exhibited a large increase in blood ascorbic acid following injection of 1 gm. on 4/17/39. At the time of injection on 5/17/39, her rumen was very distended and the injected ascorbic acid may have gone into connective tissue instead of entering the peritoneal cavity.

The observations on the remaining goats, which were fed alternately a normal diet and a vitamin C-free diet are set out in figures 2 and 3. For each animal the observations began immediately after birth of a kid and commencement of a lactation period. The level of ascorbic acid in the blood is clearly not significantly affected by the change from pasture to vitamin C-free diet or vice versa. Any slight change which may have occurred is less than the day-to-day variation. This would suggest that the goat is independent of a supply of the vitamin in its diet and is able to synthesize its own ascorbic acid unless its requirement is relatively very small. The milk of some goats may possibly contain more ascorbic acid when the animals are at pasture than when they are receiving a vitamin C-free diet, as the data for M 2 and 8 suggested; but again there is no regular close relationship between the ascorbic acid content of the milk and that of the food in the animals under observation.

## SUMMARY

1. From October, 1938 to August, 1939, 360 milk samples and 190 blood samples were taken from five Toggenburg goats receiving a normal diet and titrated for ascorbic acid content.

2. In blood the average ascorbic acid content ranged between 0.6 and 0.8 mg. per 100 cc., and in milk between 0.5 and 2.0 mg. per 100 cc. There was no consistent relationship between the content of the milk and that of the blood.

3. Following injections of 1 or 2 gm. of ascorbic acid intraperitoneally a rapid large rise in blood ascorbic acid, a rapid very large rise in urine ascorbic acid and a slower small rise in milk ascorbic acid were observed.

4. Observations on four other goats fed alternately a normal and a vitamin C-free diet indicated that the ascorbic acid of

the blood is not closely dependent on the amount of ascorbic acid in the diet, suggesting that the goat is independent of a dietary supply of ascorbic acid. The diet probably affects the ascorbic acid content of the milk more than that of the blood.

ACKNOWLEDGMENT

We wish to thank James L. Moore of the Department of Dairy Research for collecting the samples.

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# THE EFFECT OF COMPLEMENTING FACTORS ON THE QUANTITATIVE RESPONSE AND SPECIFICITY OF VITAMIN $B_6$[1]

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THREE CHARTS AND ONE PLATE (SEVEN FIGURES)

(Received for publication April 3, 1940)

Confusing and erratic animal responses encountered in studying the interrelationship and specificity of vitamin entities have been particularly prevalent in respect to those factors concerned with dermal pathology. The characteristic skin and peripheral lesions attributable to vitamin $B_6$ deficiency are seemingly difficult to obtain or reproduce with consistency in some laboratories; furthermore, relapses following the administration of vitamin $B_6$ have been difficult to explain. The indefinite distinction between acrodynia, generalized dermal symptoms and scaliness of the paws of experimental animals has likewise contributed a quota of confusion. Not only vitamin entities, but fats and certain fatty acids are involved in the problem (Burr and Burr, '29, '30; Birch and György, '36 and Birch, '38). Simplification of the basal diet and supplementation with pure entities in known amounts, as well as reduction in the multiplicity of source materials, have been of some value in facilitating an orderly integration of the relationships involved.

That the specificity of vitamin $B_6$ is dependent upon at least one other unknown factor, seems to have been well established

[1] Presented before the American Chemical Society, Division of Biological Chemistry, Cincinnati, Ohio, April 8–12, 1940.

by Lepkovsky et al. ('36), György and Eckardt ('39) and György et al. ('39). Liver and rice polish were the principal source materials used by these investigators. Bender and Supplee and co-workers (Bender and Supplee, '37; Ansbacher et al., '36; Supplee et al., '39) have also reported that acrodynia and dermal lesions in rats are prevented by a concentrate obtained from crude rice polish, and that this product supplemented with pure vitamin $B_1$ and pure riboflavin permits normal growth in rats, even after autoclaving at pH 8.5 for 5 hours at 120°C.

The availability of pure vitamin $B_6$[2] has permitted a systematic and comprehensive study of the interrelationship of this factor with other vitamin entities and concentrates of known properties. The rice polish concentrate to which reference has been made, when subjected to adsorption with fullers' earth, with or without previous removal of a substantial proportion of the protein and lipids, yields a filtrate fraction free from vitamin $B_1$, vitamin $B_6$ and riboflavin; this corroborates the observations of other investigators (Lepkovsky et al., '36; Edgar and Macrae, '37; and Oleson et al., '39). The present report contains data which interrelate the specificity of vitamin $B_6$ with a complementing and seemingly specific function of another factor contained in the treated rice polish concentrate (factor II according to Lepkovsky et al., '36) for the prevention of dermal lesions and promotion of normal growth of white rats. Nearly 1000 animals were involved in the studies reported.

## EXPERIMENTAL

Since it is the policy of these laboratories to adhere to a standardized and proved basal ration with daily supplementation of pure entities or concentrates of proved characteristics, particularly for studies involving the entities of the vitamin B complex, this regimen was followed in the present work. The basal ration consisted of the following: vitamin free casein,[3]

[2] First made available to us through the courtesy of Dr. Lepkovsky and later through the courtesy of Merck and Company.

[3] Labco Brand, distributed by the Labco Products Department, The Borden Company, New York.

20 parts; sucrose, 69 parts; hydrogenated vegetable oil,[4] 3 parts; salt mixture no. 40,[5] 4 parts; powdered agar-agar, 2 parts; and cod liver oil (medicinal grade), 2 parts. This basal dietary was further supplemented with 12.5 γ pure vitamin $B_1$ and 10 γ pure riboflavin per rat per day, fed as a dosage in standardized solution. White rats 21 to 23 days old and weighing 45 to 50 gm. at weaning were placed in individual screen-bottom cages and supplied with the basal ration only, for a period of 1 week at which time the primary supplements and variables were introduced. The variants were graded amounts of crystalline vitamin $B_6$[6] and the rice polish derivative devoid of vitamin $B_1$, vitamin $B_6$ and riboflavin, which for convenience will be designated hereinafter as "rice polish factor II."

In order to determine whether or not this rice polish derivative was free of the factors mentioned, methods previously published (Supplee et al., '38, '39) were applied for the quantitative determination of vitamin $B_1$ and riboflavin. Test quantities as high as 2.11 gm. solids of the unautoclaved rice polish factor II failed to cure the polyneuritic symptoms in ten animals. When 150 mg. per rat per day were supplied as a potential source of vitamin $B_1$ for the growth requirement, the twelve test animals lost weight during the experimental period and all were dead within 6 weeks. The rate of growth of animals receiving test quantities of 150 mg. solids per rat per day as the sole source of riboflavin was substantially the same as that previously found for animals receiving a riboflavin-free diet, namely, an average of about 2 gm. per week during an 8-week period (Supplee et al., '39). The absence of vitamin $B_6$ was established by feeding test quantities up to 300 mg. solids per day as a potential source of this factor, while maintaining the primary supplements, vitamin $B_1$ and riboflavin. An incidence of 75 to 100% uncomplicated acrodynia developed in various groups of animals of from four to thirty-two individuals per group usually within a period of 6 to 8 weeks; weight gain was stimulated at a rate of about 8 to 10 gm. per

[4] Crisco.

[5] Steenbock, H., and Nelson, E. M., J. Biol. Chem., vol. 56, p. 355, 1923.

[6] Distributed by Merck & Co., Rahway, New Jersey.

week for about 3 weeks, remaining substantially constant or slowly declining thereafter. These data, obtained with the same basal diet by merely an interchange or withdrawal of a single primary supplement, substituting the unknown test material for the factor eliminated, yield evidence which is readily interpreted without the confusion incident to the introduction of variable basal components and unknown primary supplements. Therefore, it is concluded that the rice polish factor II used for the work described hereinafter was free of vitamin $B_1$, vitamin $B_6$ and riboflavin.

Since one of the primary objects of this work was to correlate the specificity of vitamin $B_6$ with another complementing factor under controlled conditions and with recognition of the quantitative relationships between the variants, it is appropriate to mention briefly at this juncture the role which certain fats and fatty acids are known to play in the prevention of dermal lesions associated with vitamin deficiency. The basal ration employed in these studies contained fats of known and substantially uniform characteristics. A high percentage incidence of dermatitis and typical acrodynia has been obtained in hundreds of negative controls receiving this basal diet during the past 5 years. These satisfactory results, even though subject to some variations in different groups of animals from time to time seemed to warrant its continuation for the present study, in lieu of a fat-free diet, or the introduction of a more restricted fatty acid component. Comparative studies have shown that no significantly greater incidence of acrodynia has resulted from this basal diet without the 3% hydrogenated vegetable oil;[7] the introduction of 10% delays the development of acrodynia to some extent, and 20% markedly reduces the percentage incidence. Ten per cent of corn oil, cottonseed oil [8] or olive oil substituted for the hydrogenated vegetable oil markedly reduced the dermal lesions and accentuated the curative response. Butterfat and coconut oil, and the fatty acids, stearic, palmitic, capric, caproic and myristic, in amounts from 0.5 to 5% of the basal ration are seemingly without effect;

[7] See footnote 4.

[8] Wesson oil.

whereas, lard (10%), oleic and linoleic acids delay and prevent the characteristic symptoms. No significant growth or differences in growth response resulted from the various fats or fatty acids. These comparisons which need not be further detailed in view of the more extended references in the literature have served to favor the 3% hydrogenated vegetable oil basal diet as one of choice for the comparative type of investigation with which this paper is primarily concerned.

By maintaining the primary supplements constant a comparative evaluation of pure vitamin $B_6$ and the rice polish factor II and combinations of these is readily obtained. The following data contain the composite record of 735 animals made up of comparable groups during a period of about 1 year. The rice polish factor II available as a water soluble concentrate containing approximately 80% total solids was diluted to 7.5–10% solids and autoclaved for 5 hours at 120°C. after pH adjustment to 8.5. The primary supplements and vitamin $B_6$ were pure products, the vitamin $B_1$ and vitamin $B_6$ being synthetic materials, whereas the riboflavin was a pure natural product isolated from milk. It will be observed (chart 1) that vitamin $B_6$ up to 10 $\gamma$ per day in the absence of the autoclaved rice polish factor II stimulated growth for a period of only 2 to 3 weeks. The autoclaved rice polish factor II up to 100 mg. per day in the absence of vitamin $B_6$ stimulated growth to about the same degree. Neither substance in the absence of the other permitted normal development. However, when the two were combined, the vitamin $B_6$ being maintained at a constant level of 10 $\gamma$ per day, and the rice polish factor supplied in graded amounts, the growth was commensurate with the amount of the latter supplied, 100 mg. per day being adequate for a normal rate of development of about 12 to 15 gm. per week.

Table 1 shows the percentage incidence of typical and nontypical acrodynia in the animals whose growth rates are plotted in chart 1. The designation "typical acrodynia" as used for diagnostic and record purposes throughout the course of this work refers to the edematous, exudative or florid dermal lesions with loss of fur on the paws, with or without accom-

panying involvement of the nose, mouth and ears. The "non-typical acrodynia" designation refers to the exfoliative dermatitis or dry scaliness of the paws; mild or insipid cases may show this condition to only a slight degree between the toes, whereas in more extreme cases it may affect the entire area of the paws. (Figures 1, 2 and 3 show gradations in severity of this condition; all such cases have been recorded as non-typical acrodynia.)

The character and severity of the dermatitis necessary for inclusion in the records as typical acrodynia required the

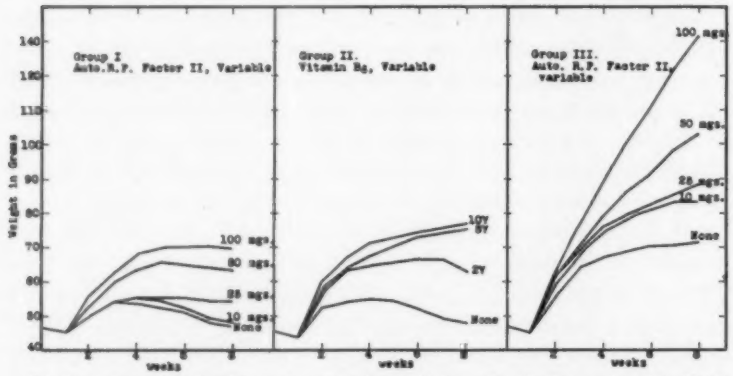


Chart 1 Growth of white rats as influenced by vitamin $B_6$ and unidentified vitamin factors in rice polish concentrate (factor II). (All animals received the basal diet only for the first week.) Groups 1 and II received 12.5 $\gamma$ thiamin and 1$\frac{1}{2}$ $\gamma$ riboflavin only per day as primary supplements; group III received 10 $\gamma$ vitamin $B_6$ daily in addition to the thiamin and riboflavin supplements.

manifestation of the florid lesions involving either the paws, or nose and mouth, or ears. While the groin and abdomen were occasionally affected, lesions in these areas were not numerous. (Figures 4, 5 and 6 show moderately severe or substantially average conditions of the paws, ears and mouth designated as typical acrodynia.) Neither generalized scaliness with exudative patches over the body nor any significant denuding has been noted during the course of these studies involving the regimen described. Ulcerated areas on the mucous lining of the lips, especially at the peripheral edges,

and on the tongue have been observed. Such cases have appeared most often on the regimen containing vitamin $B_6$ in the absence of the autoclaved rice polish factor II. Five milligrams of nicotinic acid and 200 γ of riboflavin daily for a period of 3 weeks failed to alleviate this condition; 100 mg. daily of

TABLE 1

*Incidence of dermatitis (acrodynia) in white rats as influenced by vitamin $B_6$ and unidentified vitamin factors in rice polish concentrate (factor II) (12.5 γ thiamin and 10 γ riboflavin per day as primary supplements)*

| FURTHER SUPPLEMENTS TO BASAL DIET | AMOUNT PER DAY | TYPICAL ACRODYNIA | | NON-TYPICAL ACRODYNIA (SCALY PAWS) | |
|---|---|---|---|---|---|
| | | Incidence | Av. time of appearance | Incidence | Av. time of appearance |
| | | % | *weeks* | % | *weeks* |
| Autoclaved R.P. factor II | 100 mg. | 87 | 6.4 | 63 | 4.2 |
| Autoclaved R.P. factor II | 50 mg. | 91 | 6.4 | 64 | 4.9 |
| Autoclaved R.P. factor II | 25 mg. | 96 | 5.3 | 100 | 4.4 |
| Autoclaved R.P. factor II | 10 mg. | 100 | 6.1 | 92 | 4.8 |
| None | None | 93 | 5.8 | 39 | 4.4 |
| Vitamin $B_6$ | 10 γ | 33 | 8.5 | 49 | 6.1 |
| Vitamin $B_6$ | 5 γ | 50 | 8.0 | 66 | 5.6 |
| Vitamin $B_6$ | 2 γ | 75 | 7.1 | 83 | 4.6 |
| None | None | 93 | 5.8 | 39 | 4.4 |
| Vitamin $B_6$ + auto. R.P. factor II | 10 γ 100 mg. | 0 | .. | 0 | .. |
| Vitamin $B_6$ + auto. R.P. factor II | 10 γ 50 mg. | 8 | 7.0 | 16 | 8.0 |
| Vitamin $B_6$ + auto. R.P. factor II | 10 γ 25 mg. | 12 | 8.0 | 33 | 6.0 |
| Vitamin $B_6$ + auto. R.P. factor II | 10 γ 10 mg. | 21 | 7.5 | 49 | 6.1 |
| Vitamin $B_6$ + auto. R.P. factor II | 10 γ None | 33 | 8.5 | 49 | 6.1 |

the autoclaved rice polish factor II while continuing the vitamin $B_6$ stopped the necrosis and brought about complete healing in about 4 weeks. (Figure 7 illustrates the necrotic involvement at the end of the tongue.)

The non-typical lesions in all instances preceded the more severe or typical manifestations by about 1 week to 10 days on the average. Not all of the non-typical cases progressed to

the more severe type. The percentage of the mild cases which developed into the severe form was not substantially different among those groups receiving vitamin $B_6$ in the absence of the autoclaved rice polish factor II, than when the reverse scheme of supplementation was employed. However, a lower percentage incidence of both mild and severe cases occurred among the groups receiving vitamin $B_6$ in the absence of factor II, than was the case when the autoclaved rice polish factor II was supplied in the absence of vitamin $B_6$. When both substances were supplied the animals remained free of all dermal lesions if an adequate amount of factor II was provided. Detailed consideration of the records in the literature interpreted in conjunction with the present results leads to the conclusion that the classification of the lesions obtained during the studies here reported is a logical one and that the typical and nontypical conditions are merely degrees of severity resulting from inadequate amounts, or a disbalanced relationship between vitamin $B_6$ and another complementing factor or factors contained in the autoclaved rice polish factor II.

Earlier in this paper reference was made to the variability in percentage incidence of the dermal lesions in different groups of animals from time to time. During the course of attempts to determine the cause of this variation, the following experience was encountered: A given group of animals were developing characteristic symptoms between the fourth and the fifth week in accordance with expectations. Replenishment of the basal ration required purchase of a new lot of sucrose of a different brand than had been used previously. Coincident with the use of the basal ration made from the new supply of sugar there was a remission of the symptoms in many of the animals and progress of the disease was arrested in others. Inasmuch as there had been no change in the other components of the dietary these observations seemed to incriminate the sugar. In order to test the validity of this conclusion a quantity of commercial sugar was subjected to two recrystallizations, the purified sugar being used for compounding a new batch of basal ration, and the combined mother liquors and residual sugar used for another lot of basal ration. Eighty-eight ani-

mals were involved in a comparative study of the two sugars, the primary supplements and feeding regimen being maintained as previously described. It will be noted (table 2 and chart 2) that commercial sucrose may carry impurities to a degree which affects the percentage incidence of acrodynia

TABLE 2

*Incidence of dermatitis (acrodynia) in white rats as influenced by the purity of the sucrose used in the basal ration*

*(12.5 γ thiamin and 10 γ riboflavin per day as primary supplements)*

| FURTHER SUPPLEMENTS TO BASAL DIET | AMOUNT PER DAY | INCIDENCE OF ACRODYNIA FROM RECRYSTALLIZED SUCROSE | | INCIDENCE OF ACRODYNIA FROM MOTHER LIQUOR AND RESIDUAL SUCROSE | |
|---|---|---|---|---|---|
| | | Typical | Non-typical | Typical | Non-typical |
| | | % | % | % | % |
| Vitamin $B_6$ | 10 γ | 60 | 90 | 10 | 50 |
| Vitamin $B_6$ | 5 γ | 75 | 100 | 25 | 70 |
| Vitamin $B_6$ | 2 γ | 75 | 100 | 75 | 100 |
| Autoclaved R.P. factor II | 300 mg. | 75 | .. | 25 | .. |
| Autoclaved R.P. factor II | 100 mg. | 90 | .. | 50 | .. |
| Autoclaved R.P. factor II | 50 mg. | 100 | .. | 75 | .. |

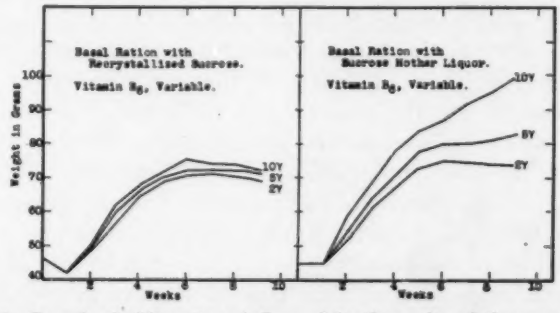


Chart 2 Growth of white rats as influenced by the purity of the sucrose used in the basal ration. (All animals received the basal diet only for the first week.) The animals in both groups received 12.5 γ thiamin, and 10 γ riboflavin and variable amounts of vitamin $B_6$ per day as primary supplements.

even under an otherwise carefully controlled dietary. This may conceivably account for the difficulties which certain laboratories experience in consistently developing the characteristic dermal lesions. György ('37) has reported that vita-

min $B_6$ is present in cane molasses, whereas beet molasses contains little or none of this factor. The offending lot of sugar encountered at this laboratory was a cane sugar.

Since there is a paucity of evidence in the literature concerning the reaction exhibited by mature animals deprived of factor II, it was deemed desirable to obtain data from such animals reared under controlled conditions. Young animals were raised to maturity on the basal ration and the supplements consisting of 12.5 γ vitamin $B_1$, 10 γ riboflavin, 10 γ vitamin $B_6$ and 150 mg. of the rice polish factor II per day for a period of 12 weeks. Two groups of twenty animals each with an equal number of males and females were used. One group received the rice polish factor II concentrate in raw, unautoclaved form, and the second group received the same material autoclaved for 5 hours at 120°C. following pH adjustment to 8.5. Following attainment in weight of about 180 gm. during the 12-week period the rice polish factor II was withdrawn, the other supplements being continued. The results from these comparisons (chart 3 and table 3) show that there was substantially no difference in rate of growth from the autoclaved and unautoclaved concentrate, all animals developing normally at a rate of 12 to 15 gm. per week. No pathological symptoms of any nature were observed. Following withdrawal of the rice polish factor II further increase in weight immediately ceased, substantially constant weight being maintained for a period of 20 weeks for those animals which survived this extended period. Survivals were greater for the group which had previously received the unautoclaved concentrate. Casualties in either of the groups were not preceded by loss of weight or emaciation; inanition and lack of vitality preceding final and unpredictable collapse constituted the characteristic reaction noted.

Acrodynia first occurred in the group which had previously received the autoclaved rice polish factor II 6 weeks following its withdrawal, whereas first occurrence did not appear until the twelfth week in the group which originally received the unautoclaved material. An increasing percentage incidence appeared in both groups with extended time. Since all animals

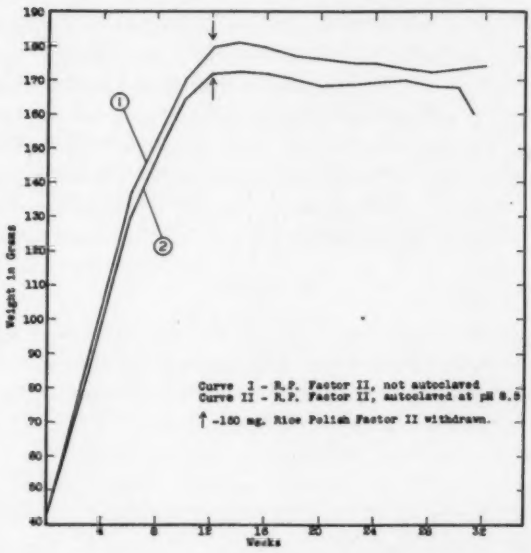
Weight in Grams
Weeks
Curve 1 - R.P. Factor II, not autoclaved
Curve II - R.P. Factor II, autoclaved at pH 8.5
↑ -150 mg. Rice Polish Factor II withdrawn.

Chart 3 Development of white rats before and after elimination of rice polish factor II. All animals received 12.5 γ thiamin, 10 γ riboflavin, 10 γ vitamin $B_6$ and 150 mg. rice polish as daily supplements for 12 weeks.

TABLE 3

*Percentage of survivors and incidence of acrodynia in mature animals following withdrawal of rice polish factor II (12.5 γ thiamin; 10 γ riboflavin; and 10 γ vitamin $B_6$ per day as primary supplements)*

| TIME AFTER WITHDRAWAL | SURVIVORS FOLLOWING WITHDRAWAL OF RICE POLISH FACTOR II | | INCIDENCE OF ACRODYNIA FOLLOWING WITHDRAWAL OF RICE POLISH FACTOR II | |
|---|---|---|---|---|
| | Not autoclaved | Autoclaved | Not autoclaved | Autoclaved |
| *weeks* | % | % | % | % |
| 6 | 100 | 100 | 0 | 10 |
| 8 | 100 | 100 | 0 | 15 |
| 10 | 100 | 100 | 0 | 15 |
| 12 | 100 | 85 | 10 | 35 |
| 14 | 85 | 45 | 25 | 35 |
| 16 | 75 | 30 | 25 | 40 |
| 18 | 55 | 25 | 40 | 50 |
| 20 | 30 | 20 | 40 | 55 |

received 10 γ vitamin $B_6$ per day throughout the entire observation period, these results are in confirmation of the evidence from young animals to the effect that the efficacy and specificity of vitamin $B_6$ for the prevention of characteristic dermal lesions are dependent upon a complementing factor or factors other than $B_1$ and riboflavin. This group of data as a whole also confirms and accentuates the evidence showing that other vitamin entities as yet unidentified are essential, not only for growth, but for prolongation of life of the mature animal.

## SUMMARY

1. A standardized basal diet is presented which, when supplemented with a controlled daily intake of thiamin and riboflavin, permits the development of acrodynia in practically 100% of the cases usually within a period of 6 to 8 weeks.

2. Vitamin $B_6$ does not permit continued growth unless an unidentified factor (or factors) present in rice polish factor II is also supplied; likewise, this complementing substance does not permit growth in the absence of vitamin $B_6$. Appropriate amounts of both substances permit normal development in the presence of thiamin and riboflavin.

3. The specificity of vitamin $B_6$ for the prevention of acrodynia and exfoliative dermatitis of the extremities is dependent upon the complementing factor or factors contained in rice polish concentrate (factor II).

4. Impurities inherent in certain lots of commercial sucrose used as the carbohydrate for the basal ration may reduce the incidence and retard the development of acrodynia in experimental animals; such impurities may also stimulate a slight degree of growth when the basal ration is supplemented with controlled amounts of thiamin, riboflavin and vitamin $B_6$.

5. Autoclaving for 5 hours at 120°C. following pH adjustment to 8.5 does not destroy the anti-acrodynia and growth-promoting substance contained in the rice polish factor II.

6. Animals grown to maturity on the synthetic ration supplemented with requisite vitamin factors failed to gain weight following withdrawal of the rice polish factor II, but did maintain constant weight for several weeks; no significant loss of

weight, emaciation or other outward symptoms preceded casualties occurring during a 20-week period following withdrawal of this factor. Inanition and lack of vitality were the only characteristic signs noted just before the final collapse. Acrodynia occurred in a substantial percentage of the animals with an increasing incidence after an extended period.

Since the preparation of this manuscript a paper by Schneider et al. (J. Biol. Chem., vol. 132, p. 539, 1940) has appeared which presents data confirming significant observations and conclusions recorded herein.

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## PLATE I

Explanation of Figures

1—Non-typical Acrodynia
(Severe Scaliness)

2—Non-typical Acrodynia
(Moderate Scaliness)

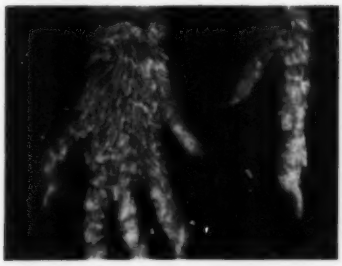
3—Non-typical Acrodynia
(Slight Scaliness)

4—Acrodynia
(Involvement of Mouth)

5—Acrodynia
(Involvement of Ears)

6—Acrodynia
(Involvement of Paws)

7—Acrodynia
(Necrosis at end of tongue)



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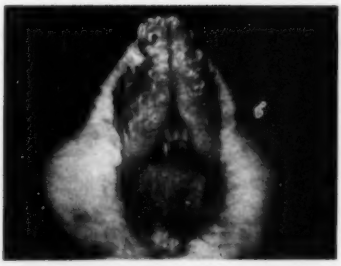

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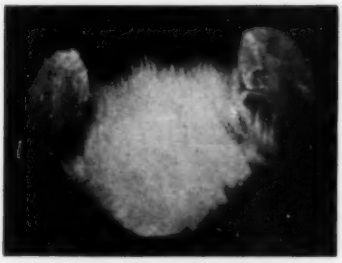

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# CEREALS AND RICKETS

## XII. THE EFFECT OF CALCIUM AND VITAMIN D ON THE AVAILABILITY OF PHOSPHORUS [1]

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(Received for publication February 26, 1940)

Investigations designed to establish an optimal ratio of calcium to phosphorus for bone calcification have resulted in divergent conclusions.[2] As it has been established in recent years that most of the phosphorus of cereal grains is present as phytic acid, which is only partially available to the animal, and since different samples of a cereal grain may vary in the kind and amount of phosphorus present (Harris and Bunker, '35) it is plausible that there may be various optimal calcium-phosphorus ratios.

The production of rickets in rats on cereal rations has been associated with an unfavorable calcium to phosphorus ratio provided, of course, that a sufficiency of each for normal performance was present. With the use of cereal free rations low in phosphorus but complete in other respects (Schneider and Steenbock, '39; Jones, '39; Day and McCollum, '39) the complications arising from the use of cereals have become more evident. Recently Krieger, Bunkfeldt and Steenbock ('40 a) using a cereal free ration reported that the utilization of phytic acid phosphorus was markedly enhanced by the addition of vitamin D. Further investigations by the same

[1] Published with the approval of the Director of the Wisconsin Agricultural Experiment Station.

[2] For references to most of the articles concerning calcium-phosphorus ratios see the paper by Lowe, Steenbock and Krieger ('39).

authors ('40 b) revealed that the calcium of calcium phytate was as readily available as that of calcium carbonate whether vitamin D was present or not. These results suggested the desirability of further work on the interrelations of calcium, phosphorus, and vitamin D with particular emphasis on determining the effect of calcium on the utilization of different forms of phosphorus. Since Zucker et al. ('22), Shelling ('25), and Shohl et al. ('28) have reported an acid-base factor in the production of rickets, due recognition was given to the possible effect of acid and basic calcium salts as well.

## EXPERIMENTAL

The basal ration employed in these experiments was essentially the same as that used in previous investigations (Krieger, Bunkfeldt and Steenbock, '40 a, '40 b), with the exception that 1.33 parts of a salt mixture low in both calcium and phosphorus was substituted for the salts used before. The composition of this salt mixture was as follows: NaCl 105, KCl 120, $MgSO_4$ 90, $MnSO_4$ 0.20, $CuSO_4 \cdot 5H_2O$ 0.39, $K_2Al_2(SO_4)_4 \cdot 24H_2O$ 0.09, NaF 0.57, KI 0.05, and Fe citrate 16.15 parts respectively. The basal ration contained 0.04% phosphorus and 0.02% calcium as determined by analysis.

The calcium and phosphorus content of the diets was adjusted as already described, the phosphorus being kept at an optimal level by the addition of either phytic acid (Krieger, Bunkfeldt and Steenbock, '40 a) or inorganic phosphates. The phosphates were added as a neutral mixture of the anhydrous mono and di potassium salts. Calcium was added as precipitated calcium carbonate, anhydrous calcium chloride, or hydrated calcium sulfate ($2\ H_2O$) to give approximate calcium-phosphorus ratios of 0.5/1, 1/1, 2/1, 4/1 and 6/1. One ration containing phytic acid phosphorus and another inorganic phosphorus, both without added calcium, were fed as negative controls. All rations were analyzed for total phosphorus (Fiske and Subbarow, '25), phytic acid phosphorus (McCance and Widdowson, '35), and calcium (Meloche et al., '33).

Viosterol, 45 U.S.P. units vitamin D per 100 gm. of ration, was used as the source of vitamin D.

The experimental conditions and criteria were the same as those used in previous investigations (Krieger, Bunkfeldt and Steenbock, '40 a, '40 b) except that calcium and phosphorus determinations on the blood sera were dispensed with. Paired feeding technique was used. The food intake was equalized

TABLE 1

*Effect of calcium on phosphorus availability at calcium-phosphorus ratios of 1/1 or less*

| RATION NO. | Ca SOURCE | P SOURCE | Ca/TOTAL P[1] | FOOD CONSUMED | CHANGE IN WEIGHT | WEIGHT OF BONE | WEIGHT OF ASH | BONE ASH |
|---|---|---|---|---|---|---|---|---|
| | | | | gm. | gm. | gm. | gm. | % |
| 1 | None | Phytic acid | 0.09 | 151 | 28.2 | 0.0931 | 0.0317 | 33.8 |
| 16 | None | Inorganic | 0.08 | 150 | 24.2 | 0.0947 | 0.0330 | 34.9 |
| 2 | $CO_3$ | Phytic acid | 0.6 | 196 | 38.7 | 0.1194 | 0.0445 | 37.4 |
| 3 | $Cl_2$ | Phytic acid | 0.6 | 195 | 38.0 | 0.1174 | 0.0442 | 37.4 |
| 4 | $SO_4$ | Phytic acid | 0.6 | 199 | 43.1 | 0.1220 | 0.0461 | 37.7 |
| 20 | $CO_3$ | Inorganic | 0.6 | 184 | 43.1 | 0.1146 | 0.0461 | 40.2 |
| 31 | $SO_4$ | Inorganic | 0.5 | 176 | 49.5 | 0.1203 | 0.0469 | 39.0 |
| 5 | $CO_3$ | Phytic acid | 1.0[2] | 221 | 55.0 | 0.1157 | 0.0443 | 38.2 |
| 6 | $Cl_2$ | Phytic acid | 1.0[2] | 225 | 56.0 | 0.1373 | 0.0515 | 37.2 |
| 7 | $SO_4$ | Phytic acid | 1.0[2] | 221 | 57.0 | 0.1249 | 0.0477 | 37.1 |
| 17 | $CO_3$ | Inorganic | 1.0[2] | 216 | 54.3 | 0.1404 | 0.0611 | 43.4 |
| 29 | $Cl_2$ | Inorganic | 0.9[2] | 209 | 59.4 | 0.1424 | 0.0607 | 42.6 |
| 32 | $SO_4$ | Inorganic | 1.0[2] | 208 | 50.0 | 0.1328 | 0.0606 | 45.0 |

[1] The calcium phytic acid phosphorus ratio was practically identical.

[2] At these ratios three rats in one group died from an unknown cause after 3 weeks. Three rats were accordingly also taken for analysis at this time from the other groups leaving only three for the final analysis. The results from both were in harmony. All other analyses were based on six rats taken at 4 weeks.

between rats on identical calcium-phosphorus ratios after the first week. Each feeding experiment was terminated at the end of 4 weeks unless otherwise indicated.

## RESULTS

It is evident from the data (tables 1 and 2) that a change in the level of calcium produced changes in growth, food consumption, weight of bone, weight of bone ash, and percentage of bone ash. In the negative control groups (table 1) there

were no differences in calcification regardless of the form of phosphorus given. It is quite apparent that here calcium was the limiting factor. When the calcium was increased to a calcium-phosphorus ratio of 0.5/1 there resulted a small but definite improvement in growth, food consumption, and calcification—but calcium was still the limiting factor. Slightly better calcification resulted with inorganic phosphorus than with phytic acid phosphorus although less ration was consumed.

TABLE 2

*Effect of calcium on phosphorus availability at calcium-phosphorus ratios of 2/1, 4/1 and 6/1*

| RATION NO. | Ca SOURCE | P SOURCE | Ca/ TOTAL P[1] | FOOD CONSUMED | CHANGE IN WEIGHT | WEIGHT OF BONE | WEIGHT OF ASH | BONE ASH |
|---|---|---|---|---|---|---|---|---|
| | | | | gm. | gm. | gm. | gm. | % |
| 8 | $CO_3$ | Phytic acid | 1.9 | 237 | 64.3 | 0.1125 | 0.0328 | 29.1 |
| 9 | $Cl_2$ | Phytic acid | 1.8 | 231 | 55.0 | 0.0991 | 0.0224 | 22.8 |
| 10 | $SO_4$ | Phytic acid | 2.0 | 235 | 57.5 | 0.1103 | 0.0329 | 29.5 |
| 21 | $CO_3$ | Inorganic | 1.9 | 214 | 53.2 | 0.1419 | 0.0636 | 44.8 |
| 30 | $Cl_2$ | Inorganic | 1.7 | 217 | 59.2 | 0.1458 | 0.0673 | 45.8 |
| 33 | $SO_4$ | Inorganic | 1.8 | 216 | 56.9 | 0.1488 | 0.0672 | 45.1 |
| 11 | $CO_3$ | Phytic acid | 3.8 | 208 | 43.0 | 0.0944 | 0.0202 | 21.4 |
| 13 | $SO_4$ | Phytic acid | 3.8 | 209 | 45.6 | 0.0975 | 0.0239 | 24.4 |
| 18 | $CO_3$ | Inorganic | 4.3 | 215 | 51.6 | 0.1349 | 0.0569 | 42.0 |
| 34 | $SO_4$ | Inorganic | 3.6 | 210 | 51.7 | 0.1368 | 0.0626 | 45.7 |
| 14 | $CO_3$ | Phytic acid | 5.5 | 213 | 42.7 | 0.0964 | 0.0206 | 21.5 |
| 15 | $SO_4$ | Phytic acid | 5.9 | 215 | 48.9 | 0.0985 | 0.0241 | 24.4 |
| 19 | $CO_3$ | Inorganic | 5.7 | 218 | 52.8 | 0.1339 | 0.0553 | 41.1 |
| 35 | $SO_4$ | Inorganic | 5.3 | 217 | 49.2 | 0.1351 | 0.0604 | 44.1 |

[1] The calcium phytic acid phosphorus ratio was practically identical.

When the calcium intake was increased to a calcium-phosphorus ratio of 1/1 no further increase in calcification resulted with phytic acid, although food consumption and growth continued to increase. However, with inorganic phosphorus better calcification resulted which was concomitant with better food consumption and better growth.

When the calcium content of the ration was increased still more to a calcium-phosphorus ratio of approximately 2/1 (table 2) a marked decrease in bone ash resulted with phytic

acid although there was little effect on food consumption or growth. On the other hand, inorganic phosphorus increased the percentage of bone ash slightly. Apparently calcium was no longer limiting calcification in the inorganic groups.

Further increases in the calcium content of the rations to calcium-phosphorus ratios of approximately 4/1 and 6/1 produced even more drastic changes than the 2/1 ratio. The availability of the phytic acid phosphorus was decreased still further but apparently reached the limit at the 4/1 ratio. These same levels, however, had little effect on the utilization of the inorganic phosphorus.

Variations in the source of the calcium produced small but persistent differences in the utilization of both forms of phosphorus, particularly at the higher ratios. Up to a calcium-phosphorus ratio of 1/1, where calcium obviously was the limiting factor, it was without effect (table 1). However, when the calcium was increased to a 2/1 ratio, the level being then slightly in excess of optimal (Krieger, Bunkfeldt and Steenbock, '40 b), the potentially acid salt, calcium chloride, produced a lower bone ash than the carbonate or the sulfate (table 2). This however was only true with phytic acid. With inorganic phosphorus no difference was observed. The carbonate gave the same result as the sulfate but when the calcium was increased to ratios of 4/1 and 6/1 the neutral salt, calcium sulfate, produced a slightly higher bone ash than the carbonate regardless of the source of the phosphorus. Calcium chloride was not used at these higher ratios, as some of the rats ate sparingly of these rations and failed to grow.

An acid-base effect has been observed before. Zucker et al. ('22) and Shelling ('25) reported an increased severity in rickets on acid diets and Shohl et al. ('28) reported good ash deposition with neutral diets, less with alkaline diets, and least with acid diets. On the other hand, McCollum et al. ('22) as well as Mellanby ('25) reported the acid-base factors as unimportant. Undoubtedly there are other factors such as solubility of the calcium salts which must be given due recognition.

The addition of vitamin D to rations of varying calcium-phosphorus ratios revealed differences in the utilization of phytic acid as contrasted with inorganic phosphorus (table 3). No difference was revealed when vitamin D was added to the negative control rations regardless of the source of the phosphorus. However, at the 2/1 ratios vitamin D markedly

TABLE 3

*Effect of calcium and vitamin D[1] on phosphorus availability at various calcium-phosphorus ratios*

| RATION NO. | Ca SOURCE | P SOURCE | Ca/TOTAL P[2] | FOOD CONSUMED | CHANGE IN WEIGHT | WEIGHT OF BONE | WEIGHT OF ASH | BONE ASH (PLUS D) | BONE ASH (MINUS D)[3] |
|---|---|---|---|---|---|---|---|---|---|
| | | | | *gm.* | *gm.* | *gm.* | *gm.* | % | % |
| 1A | None | Phytic acid | 0.09 | 152 | 16.3 | 0.1116 | 0.0393 | 35.3 | 33.8 |
| 16A | None | Inorganic | 0.08 | 152 | 19.5 | 0.1063 | 0.0357 | 33.7 | 34.9 |
| 8A | $CO_3$ | Phytic acid | 1.9 | 235 | 57.1 | 0.1896 | 0.0924 | 48.3 | 29.1 |
| 9A | $Cl_2$ | Phytic acid | 1.8 | 235 | 48.0 | 0.1403 | 0.0629 | 44.7 | 22.8 |
| 10A | $SO_4$ | Phytic acid | 2.0 | 235 | 59.4 | 0.1733 | 0.0865 | 49.9 | 29.5 |
| 21A | $CO_3$ | Inorganic | 1.9 | 235 | 61.0 | 0.1973 | 0.1120 | 56.4 | 44.8 |
| 30A | $Cl_2$ | Inorganic | 1.7 | 235 | 64.9 | 0.2077 | 0.1137 | 54.8 | 45.8 |
| 33A | $SO_4$ | Inorganic | 1.8 | 235 | 65.7 | 0.2087 | 0.1313 | 54.9 | 45.1 |
| 11A | $CO_3$ | Phytic acid | 3.8 | 208 | 30.9 | 0.1218 | 0.0502 | 41.2 | 21.4 |
| 13A | $SO_4$ | Phytic acid | 3.8 | 208 | 36.8 | 0.1422 | 0.0659 | 46.5 | 24.4 |
| 18A | $CO_3$ | Inorganic | 4.3 | 208 | 44.6 | 0.1987 | 0.1065 | 53.5 | 42.0 |
| 34A | $SO_4$ | Inorganic | 3.6 | 208 | 47.9 | 0.2047 | 0.1134 | 55.3 | 45.7 |

[1] Forty-five U.S.P. units vitamin D (viosterol) per 100 gm.

[2] The calcium-phytic acid phosphorus ratio was practically identical.

[3] Values from identical rations minus vitamin D taken from tables 1 and 2.

improved the utilization of both forms of phosphorus, inorganic phosphorus producing the most bone ash but the phytic acid phosphorus giving the largest increment. It must be noted that the possible increment of improvement which could be achieved with inorganic phosphorus was definitely limited by the higher bone ash produced without vitamin D.

When the calcium-phosphorus ratio was increased to 4/1 the effect of vitamin D was still evident, but to only a slight degree with phytic acid. The increase in calcium caused a slight decrease in the percentage of bone ash of these latter groups over those on a lower ratio even though vitamin D was present. This effect was not observed with inorganic phosphorus. Apparently the calcium-phosphorus ratios are of no great importance when the phosphorus is present in the inorganic form.

Variations in the source of calcium again produced small but persistent differences in calcification even in the presence of vitamin D (table 3). At the 2/1 ratio calcium chloride produced less bone ash than the sulfate or the carbonate. At the 4/1 ratio the carbonate produced less bone ash than the sulfate, regardless of the source of phosphorus.

## SUMMARY

In feeding experiments with rats the availability of phytic acid phosphorus was markedly affected by the calcium and vitamin D content of the diet. With an optimal intake of phosphorus and a calcium-phosphorus ratio of 1/1 phytic acid phosphorus was almost as readily utilized as the inorganic form. However, with an increase in the calcium-phosphorus ratio to 2/1 the availability of phytic acid phosphorus was markedly decreased. Further increases in the calcium content of the rations decreased the availability of phytic acid phosphorus still more. On the other hand, inorganic phosphorus was not markedly affected by an increase in calcium even up to a ratio of 6/1.

Different sources of calcium had a small but definite differential effect on the utilization of phosphorus. Calcium chloride produced a more severe rickets than either the carbonate or the sulfate. Calcium sulfate produced more bone ash than calcium carbonate.

Vitamin D improved the utilization of both forms of phosphorus. This effect was more pronounced with phytic acid since the increment of improvement possible within the limits of calcification was less with the inorganic form. Even in

the presence of vitamin D phytic acid phosphorus was not so readily available as the inorganic form. The aforementioned relative effects of different sources of calcium were also noted in the presence of vitamin D.

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# THE BIOLOGICAL ASSAY OF RIBOFLAVIN [1]

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TWO FIGURES

In bioassays for riboflavin in which the growth of the rat is used, one of the most important problems is the choice of material to use as a source of the vitamin B-complex. This substance must be free of riboflavin so that the rats may be depleted of this vitamin, but adequate with respect to all other components of the vitamin B-complex. The object of the present study was to compare three such materials already widely used for this purpose as supplements to a purified diet, viz., extracts of rice polishings (Cook et al., '37; Day et al., '37; Helmer, '37; Supplee et al., '39), whole wheat (Bourquin and Sherman, '31) and yeast (Copping and Roscoe, '37). Having determined which of these was the most satisfactory for assays, it was our aim to set up criteria for depletion and to establish the relationship between graded doses and the gain in weight of rats depleted of riboflavin only. The final purpose of this study was to apply the information obtained to the performance of bioassays.

## EXPERIMENTAL

Albino rats of the Yale strain, maintained on a satisfactory colony diet (The Vitamin Conference, U. S. P. XI, '31) were used. At the age of 16 to 19 days, when the young rats weighed

[1] A preliminary report was presented before the Pacific Division, American Association for the Advancement of Science at Stanford University, California, June 29, 1939.

between 30 and 40 gm., litters were transferred to a diet free of water-soluble vitamins which is described below. At 21 days the young were weaned and the mothers returned to the stock ration. The litters were transferred to individual cages having raised screen floors and continued on the basal diet plus daily doses of all the vitamins of the vitamin B-complex except riboflavin, administered in small dose cups. The rats were weighed three times weekly until depleted. Each experiment consisted of several groups of ten or more rats, distributed as uniformly as possible with regard to sex and litter. Every rat in a group received the same supplements.

The basal diet consisted of: corn starch,[2] 64%; casein,[3] 20%; hydrogenated cotton seed oil,[4] 10%; salt mixture,[5] 4%; cod liver oil,[6] 2%. This diet as well as water, U.S.P., was supplied ad libitum. Vitamins, other than riboflavin which was provided after depletion, and A and D which were included in the diet, were given separately throughout the depletion and assay periods. Vitamin $B_1$ was supplied as thiamin chloride,[7] 48 μg. daily. This large dose was considered necessary in order to minimize the variation of this factor, present in different quantities in the extracts under investigation, and to provide a sufficient amount so that vitamin $B_1$ would not be a limiting factor for growth even when large doses of flavin were given in an assay. The aggregate of thiamin given, approximately 24 international units per rat per day in all groups, approached the maximum dose for growth (Waterman and Ammerman, '35) and storage (Leong, '37). Solutions of thiamin were prepared according to the procedure in U. S. Pharmacopoeia XI, 1939 supplement.

The extracts used as sources of the water-soluble vitamins except riboflavin, were prepared as follows. Whole wheat

[2] Duryea's improved.
[3] Labco brand, vitamin-free.
[4] Crisco.
[5] Osborne and Mendel (no. 1 of U.S.P. XI).
[6] U. S. P.
[7] Merck.

extract was made according to the method of Bourquin and Sherman ('31) except that it was not dried on corn starch nor incorporated in the diet, but fed separately in daily doses so that it could be compared with other materials similarly administered. The aqueous alcoholic extract of 5 kg. of whole wheat was distilled under reduced pressure until the volume was 1 liter. The rice polishings concentrate [8] was the aqueous acetone extract described by Cook and Carroll ('36). This was used untreated or after one treatment with a small amount of fuller's earth. Each 100 gm. of rice polishings concentrate was diluted with 1.4 liters of phosphate-citric acid buffer at pH 4.2; 12 gm. of fuller's earth was added and the mixture was stirred for 15 minutes, filtered and evaporated in a partial vacuum at 50°C. For the yeast extract, two commercial yeast preparations were used after treatment with fuller's earth to remove riboflavin. Each 100 gm. of yeast extract was diluted with 2 liters of phosphate-citric acid buffer at pH 4.2; 30 gm. of fuller's earth was added. Sample A was treated with one lot of fuller's earth, sample B with two. Evaporation was carried out as for the rice polishings concentrate. The conditions chosen for adsorption, the amount of adsorbent, pH and time of treatment, were not those ordinarily used to effect the removal of either thiamin or vitamin $B_6$.

When rats had reached a plateau in their individual growth curves, they were considered depleted. All depleted animals fell into one of two categories, (a) those whose body weights were maintained for 1 week within 3 gm. and (b) those which had lost more than 3 gm. on 2 successive weighing days. The shortest depletion time allowed was 7 days after weaning. Some of the rats included in the assays were growing at a very slow rate, since a gain of 3 gm. in the week preceding an assay was permitted. Some of the negative control rats, continued for 4 weeks after depletion on the supplements without added riboflavin (rice polishings concentrate and thiamin only), grew slowly also, suggesting that the supplement used might have contained traces of riboflavin. How-

[8] 'Ryzamin-B' Burroughs Wellcome & Co. (U. S. A.) Inc.

ever, the prompt and sustained response to doses of riboflavin as small as 2.5 μg. daily shows clearly that the plateau was related to a deficiency of this vitamin. The question of the effect of arbitrary limits set for depletion time upon the results of experiments will be considered subsequently.

The criteria selected for evaluation of the extracts used as sources of the B-complex in riboflavin assays were (a) the time required for depletion and (b) the growth following the administration of a so-called "maximum dose" of pure riboflavin to rats depleted on the basal diet supplemented with the extracts under investigation. The first (a) should indicate whether the extract is free from riboflavin and second (b) should indicate whether the extract is adequate with respect to all other components of the vitamin B-complex.

The mean values for depletion time for rats on the supplements selected varied from 13 to 34 days (table 1). No significant differences [9] in mean depletion time were found when the value for the group receiving 150 mg. of "treated" rice polishings concentrate was compared with the values for all other groups, except the group receiving 150 mg. of yeast extract, sample A. In the latter case the time was significantly longer. The next group also, fed the same amount of another yeast extract had an even more extended depletion period. Using small groups of rats no significant shortening of the time required for depletion was evident when "treated" rice polishings concentrate was substituted for the "untreated" preparation (table 1). However, when larger numbers were used (table 1, last 3 assays) proof [10] that the treatment by adsorption significantly shortened the depletion time was adduced. Here the maximum time allowed for

[9] When the statistic $t$ corresponded to a probability, P, equal to or less than 0.05, it was concluded that the mean of the group in question differed significantly from the mean of the group receiving 150 mg. of "treated" rice polishings concentrate. Fisher's tables ('38) were used.

[10] Here $t$ corresponded to a value of P less than 0.01 when the mean of either group receiving "treated" rice polishings concentrate was compared with the mean of the group receiving the same amount of the untreated preparation.

depletion was 28 days when "treated" rice polishings concentrate was used, 56 days when "untreated" was used; some rats failing to show depletion within the time thus set were not used in the assay and not included in the figures quoted. About 25% were excluded because of the time limit when "untreated" rice polishings concentrate was used; less than 5% when the "treated" preparation was employed.

TABLE 1

*Time required for depletion of riboflavin*

| SOURCE OF B-COMPLEX FACTORS [1] | NUMBER OF RATS | DEPLETION TIME REQUIRED (DAYS AFTER WEANING) | |
|---|---|---|---|
| | | Mean | Standard deviation |
| 150 mg. rice polishings concentrate, "treated" | 13 | 16 | ± 8.5 |
| 150 mg. rice polishings concentrate, "untreated" | 10 | 21 | ±10.3 |
| 75 mg. rice polishings concentrate, "untreated" | 10 | 15 | ± 2.8 |
| 600 mg. extract of whole wheat | 10 | 13 | ± 4.7 |
| 300 mg. extract of whole wheat | 10 | 14 | ± 2.3 |
| 150 mg. yeast extract, sample A | 10 | 29 | ± 8.2 |
| 150 mg. yeast extract, sample B | 14 [2] | 34 | |
| 75 mg. yeast extract, sample B | 12 | 16 | ±11.0 |
| 150 mg. rice polishings concentrate, "treated" | 59 [3] | 13.1 | ± 2.5 |
| 150 mg. rice polishings concentrate, "treated" | 60 [3] | 17.0 | ± 8.9 |
| 150 mg. rice polishings concentrate, "untreated" | 47 [4] | 26.9 | ±14.0 |

[1] Each rat received crystalline thiamin in addition.

[2] Of this group, 29% were not depleted by the eighth week and were discarded. The mean includes these rats, assuming their depletion time to be 56 days, but the standard deviation was not determined.

[3] Rats were excluded from these assays when their depletion time was longer than 28 days.

[4] Rats were excluded fom this assay when their depletion time was longer than 56 days.

The second criterion used in judging the adequacy of the extracts employed as sources of the B-complex except riboflavin was the growth response, i.e., gain during a 4-week period after depletion when a "maximum dose" of riboflavin [11] was administered (fig. 1). This dose is defined as the smallest amount of riboflavin administered daily which will

[11] Labeo brand. Solutions were prepared in sodium citrate buffer containing 25% alcohol at pH 5.0.

produce growth at a maximum rate in depleted rats; it has already been found (Edgar et al., '37) to be about 40 μg. When the five groups receiving 75 or 150 mg. of rice polishings concentrate, or 150 mg. of yeast extract (sample A and sample B) were compared, no significant difference [12] in gain during 4 weeks was found. The growth rate was significantly less than that of the above five groups when only 75 mg. of yeast extract was given, or when either of the two doses of wheat extract was used. The growth of rats given supplements which allowed the most rapid gains was compared with that occurring in our colony fed a mixed ration. The females

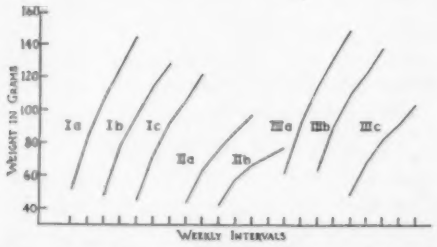


Fig. 1 Growth response in 4 weeks to a daily dose of 40 μg. of riboflavin. The sources of other B-complex factors were: I Rice polishings concentrate, *a* 150 mg., *b* 150 mg. "treated," *c* 75 mg.; II Wheat extract, *a* 600 mg., *b* 300 mg.; III "Treated" yeast extract, *a* 150 mg. sample A, *b* 150 mg. sample B, *c* 75 mg. sample B. Riboflavin was removed by adsorption from the samples labeled "treated."

in the experiment with 150 mg. of rice polishings concentrate were growing at a rate not significantly different from that of colony females for a corresponding 4-week period, whereas the males in the experiment were growing at a rate significantly less.

Since the responses of groups of rats to a 40 μg. dose of riboflavin, when the B-complex was provided by the wheat extract (either 300 or 600 mg. per day), was significantly less than that of all groups receiving rice polishings concentrate including the 75 mg. level, it was obvious that some B-complex

[12] The statistic *t* corresponded to a probability, P, greater than 0.05.

factor was limiting their growth. Under the conditions of our experiments, 600 mg. of the extract of wheat was not adequate for growth at a maximum rate. In the group receiving only 300 mg. of this extract, two rats developed florid dermatitis of the ears, like that usually ascribed to vitamin $B_6$ deficiency during the fourth week on the riboflavin dose. The swollen, scaly, inflamed condition of these ears disappeared in 2 weeks after changing the dose from 300 mg. of wheat extract to 75 mg. of rice polishings concentrate, the other supplements remaining the same. By this procedure the growth rate was doubled also. Evidence as to the presence of vitamin $B_6$ in curative amounts in the doses of rice polishing concentrate employed in our study is indicated by this experiment.

Although in equal doses yeast extract and rice polishings concentrate allowed approximately equal responses when a maximum dose of riboflavin was given (fig. 1), the fact that the groups given the yeast preparations (even after treatment with fuller's earth) had very long depletion times (table 1) was not overlooked when the final selection of the material most suitable for riboflavin assays was made.

Criticism might be made of the use of adsorption to remove riboflavin from rice polishings concentrate in view of the possibility that other essentials might be removed simultaneously even with the conditions carefully selected to avoid this possibility. Evidence that this is not the case is contributed as follows: (a) rapid growth, nearly equal to that of our colony, was obtained when only pure riboflavin was given in maximum dosage, (b) no symptoms appeared except those clearly due to riboflavin deficiency (described below) and (c) the administration of the vitamin most likely to be removed under our conditions of adsorption, namely vitamin $B_6$, to rats receiving all other vitamins including riboflavin, produced no difference in growth rate (table 2). Evidence as to the presence of adequate amounts of vitamin $B_6$ in the rice polishings concentrate employed in our study has been reported elsewhere (du Vigneaud et al., '39).

Having established the fact that rice polishings concentrate after one treatment by adsorption with fuller's earth was a satisfactory source of the vitamin B-complex components other than riboflavin in that it allowed a reasonably short depletion time when a dose was employed which would permit maximum growth, this material was used in an experiment in which the response of rats to graded doses of riboflavin was studied. The doses selected were 2.5, 5, 10, 20 and 40 μg. of riboflavin. There were ten rats in each group, five males and five females. Equations for regression lines relating the mean response, increase in weight in 4 weeks, to the logarithm of the dose, were calculated. The curves, drawn separately for males and females, seemed to be linear up to

TABLE 2

*Results of experiment in which additional vitamin $B_6$ was used*

| SUPPLEMENTS | NUMBER OF RATS | GAIN IN WEIGHT, 4 WEEKS | |
|---|---|---|---|
| | | Mean | Standard deviation |
| | | *gm.* | *gm.* |
| 150 mg. "treated" rice polishings concentrate + 48 μg. thiamin + 10 μg. riboflavin | 10 | 55.4 | ± 9.42 |
| Same daily supplements + 200 μg. vitamin $B_6$[1] per week | 10 | 56.0 | ±10.21 |

[1] Merck.

the 20 μg. dose, and proof for their linearity up to this dose level was adduced by methods outlined in Snedecor's text ('38). At dose levels higher than 20 μg., the values for both males and females indicate that the dose-response curves tend to flatten (compare Edgar et al., '37), although the point for the male response on the 40 μg. dose does not depart significantly from the linear relationship established by the calculated regression line.

Examination of the slopes of the regression lines for males and for females, up to the 20 μg. dose (fig. 2), shows that these are similar, the slope of the males being slightly steeper. The equations representing these lines are:

$$y = -2.64 + 71.24x \text{ (males)},$$
$$y = 1.64 + 68.66x \text{ (females)},$$

where y is the gain in weight in 4 weeks and x is the logarithm of the dose in micrograms. A comparison of the two slopes by means of a method described by Fisher ('38) shows that no significant difference in slope was established in this experiment ($P = 0.7$). Lindholm ('38) reports a considerably steeper slope for the regression line for the males than that for the females.

The only symptoms frequently observed in the nearly 500 rats used in these experiments were those of the eye. Only

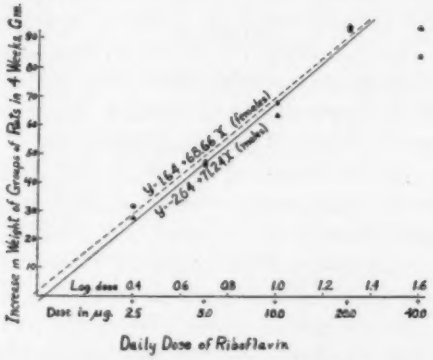
Increase in Weight of Groups of Rats in 4 Weeks Gm.
y=16.4+68.66 X (females)
y=2.64+71.24 X (males)
Log dose 0.4 0.6 0.8 1.0 1.2 1.4 1.6
Dose in μg. 2.5 5.0 10.0 20.0 40.0
Daily Dose of Riboflavin

Fig. 2 Curves of response relating the logarithm of the dose to the increase in weight in 4 weeks: △ = mean response for males; ○ = mean response for females. The response to the 40 μg. dose (log of 40 = 1.6) was not used in calculating the equations given.

four cases of cataract were noted. Many rats, however, exhibited the blepharitis, alopecia of the lids, photophobia and lacrimation previously described (Day et al., '31; Bourne and Pyke, '35). The inflammation never appeared until after the third week of depletion, and was found most frequently in the negative controls although some rats receiving small doses of riboflavin also exhibited the symptoms. In one experiment where 130 rats were used, 30% of the negative controls, 24% of those receiving doses equivalent to 2.5 to 8 μg. and 5% of those receiving 10 μg. of riboflavin had inflamed eyes or eyelids. Since no microscopic study was

made, it is not certain whether vascularization of the cornea specific for riboflavin deficiency (Bessey and Wolbach, '39) developed.

A number of rats showing eye symptoms were treated with large doses of a "filtrate factor" concentrate or vitamin A (cod liver oil) to determine whether the condition was curable by these factors. Cures were not effected. However, when a dose of 40 μg. of riboflavin was given daily, improvement began immediately, and in every case cures were complete in about 2 weeks. El-Sadr ('39) described similar cures with pure riboflavin in rats receiving liver or yeast preparations as sources of the B-complex. The occurrence of specific riboflavin deficiency symptoms and their cure under our experimental conditions provides justification for the use of the non-specific growth method for assay purposes.

Using the method outlined in this paper, riboflavin containing materials, principally yeast concentrates, have been assayed. Throughout the assays the basal diet supplemented daily with 150 mg. of "treated" rice polishings concentrate and 48 μg. of thiamin was given to rats of weight and age specified earlier in this paper. When the graphic method was used in interpreting results two control groups were given doses of riboflavin of such size that the mean responses lay on the linear portion of the dose-response curve. An attempt was made to select a dose of the "unknown" so that the response would fall between the responses of the two control groups. When an algebraic method was employed to interpret the results by calculation of the regression lines, two or three groups for both control and "unknown" were included in the assay, the doses being in the ratio of 1:2 or 1:2:4. In several assays set up in this way, each group comprising ten rats, the limits of error within which the potency could be expected to fall in twenty-one trials out of twenty-two, were found to be approximately 79 and 127%. By setting a limit on the time for depletion, i.e., excluding rats not depleted in 28 days, the accuracy of the assays was increased somewhat. In a group of assays without the time

limitation, the average standard deviation for gain in weight in 4 weeks was 15.9 gm.; in another group of assays with this limitation, the average standard deviation was 9.5 gm. Other devices for decreasing the limits of error of the assay such as increasing the number of rats per group, or the number of groups may be employed.

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## SUMMARY

A study of the rat growth method for the biological assay of riboflavin has been made. Comparisons of extracts of rice polishings, whole wheat and yeast, used as sources of the vitamin B-complex other than riboflavin in these assays, indicated that the rice polishings concentrate after one adsorption with fuller's earth is the most satisfactory supplement. Under the experimental conditions employed, neither the wheat extract nor the yeast extract satisfied the two requirements as well as did rice polishings concentrate, namely, freedom from riboflavin and adequacy with respect to all other components of the vitamin B-complex. It was established that the relationship between the logarithm of the dose and the response, gain in weight in 4 weeks, is linear up to the 20 μg. dose and that the regression lines for males and females are practically identical. Methods whereby the results thus described may be utilized in the estimation of the riboflavin content of unknowns and the limits of error of such assays are suggested.

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# THE EFFECTS PRODUCED BY DECREASING THE CALCIUM AND PHOSPHORUS INTAKE ON CALCIUM AND PHOSPHORUS ABSORPTION AND DEPOSITION AND ON VARIOUS BODILY CONSTITUENTS OF THE RAT

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An excess of calcium and phosphorus in the diet of albino rats was found in a previous study (Haldi, Bachmann, Wynn and Ensor, '39) to result in a significant diminution of the fat content of the body, an increase in the percentage water and the renal lesions that had been previously described by MacKay and Oliver ('35) and attributed by them to the high intake of phosphate. The present investigation was undertaken to determine the effects of lowering the calcium and phosphorus intake on the calcium and phosphorus balance and on the same bodily constituents as in the previous study. In addition the composition of the bone was also studied.

## PROCEDURE

The general procedure of these experiments was the same as in the former investigation (Haldi et al., '39). The animals were selected at weaning in groups of three of the same sex from the same litter and were approximately the same weight. They were fed the Wistar diet for 1 week, each animal receiving the same amount of food as its litter mate. They were then placed on the experimental rations. The basal or control ration, which will be designated as ration 1, consisted of 72% sucrose, 18% casein, 6% yeast and 4% of the

Osborne-Mendel salt mixture ('19), which was slightly modified by reducing the calcium carbonate and phosphoric acid content so that the ration contained 1% calcium and 0.57% phosphorus. The other two rations, to be referred to as rations 2 and 3, were of the same composition except for the salt mixture which was changed with respect to calcium and phosphorus so that rations 2 and 3 contained approximately 67% and 40%, respectively, as much calcium and phosphorus as ration 1. The average calcium content of the three rations was, respectively, 1.0, 0.67, and 0.40% of the food mixture and the phosphorus content 0.57, 0.39, and 0.24%. The amount of calcium and phosphorus present in the mixture was determined by analysis. The Ca/P ratio in the three rations ranged from 1.7 to 1.8. Eight males and the same number of females were placed on each of the three rations. A 6:1 mixture of cod liver oil and linoleic acid was administered daily to each animal by medicine dropper. The daily intake of vitamin A was approximately 1350, and of vitamin D, 25 international units.

The food intake of the three litter mates in each group was maintained as nearly equal as possible from day to day and completely equalized once a week. The caloric value of the total food intake of litter mates on the three rations was therefore the same at the conclusion of the experiment, although there was a slight difference in the bulk of the food mixture that was ingested because of the difference in the salt content. The average total amount of food consumed, exclusive of the salt mixture, was 847 gm. for the males and 660 gm. for the females. The feces were collected throughout the experiment and analyzed for calcium and phosphorus.

When the animals had been on the experimental ration for 70 days, they were fasted for 24 hours with free access to water, then decapitated, ground to a homogeneous mass and analyzed for water, fat, protein, calcium, and phosphorus in the manner described elsewhere (Haldi, Bachmann, Wynn and Ensor, '39). Before the bodies were ground, the right femur was removed and weighed and subsequently dried in

an oven at 110°C. The bone was then freed from organic material by the ashing method of Gabriel as modified by Morgulis ('31), dissolved in HCl and analyzed for calcium and phosphorus.

## RESULTS

In order to facilitate a comparison of the results of these experiments with those obtained with a larger calcium and phosphorus intake, the data are presented in the same tabular form as in the earlier report. The probability of the differences between averages being due to chance, as shown in table 3 for the various body constituents and designated in the text as P was determined by applying the t test of Fisher ('32) using the formula for t which takes into account the correlation between litter mates.

*Calcium and phosphorus absorption.* The difference between the amounts of calcium and phosphorus ingested and those recovered in the feces will be referred to for descriptive purposes as the "amount absorbed." The objective of these experiments makes it unnecessary to take into account the excretion by the intestinal wall.

The data in table 1 show that with a progressive decrease in the calcium and phosphorus intake there was a progressive decrease in absorption. Although absorption and deposition diminished with the smaller intake, part of the ingested calcium and phosphorus was excreted in the feces. The average fecal calcium values on the three rations proved to be 66%, 57% and 34%, respectively, of the calcium intake. The phosphorus in the feces was, respectively, 55%, 46% and 33% of the amount ingested. It is impossible to state whether the higher percentages in the case of calcium excretion are to be accounted for by a smaller absorption of calcium than of phosphorus or by a larger excretion of calcium through the intestinal wall.

*Absorption of food material.* The organic matter in the feces of the males and females, as determined by subtracting the total ash from the dry weight, was 26, 23 and 20 gm.,

respectively, on the three rations. With the decrease in the calcium and phosphorus content of the diet, there was therefore a slight increase in the amount of food material absorbed. The difference, however, of 6 gm. in absorption on diets 1 and 3 was probably inconsequential as it was less than 1% of the total organic material ingested throughout the experiment.

*Urinary excretion of calcium and phosphorus.* Direct determination of the urinary excretion of calcium and phosphorus by analysis of the urine collected from day to day was impracticable under the conditions of the experiment. The food mixture was kept in conical cups to safeguard against spilling, but a small amount would adhere to the front feet which were almost invariably inserted into the cups when the animals were feeding. Although the amount of the food mixture lost in this way was inconsequential it was thought that it would be sufficient to vitiate the results of urinary analysis since the amount of calcium and phosphorus excreted daily in the urine was extremely small.

It is possible, however, to determine approximately the quantity of calcium and phosphorus excreted in the urine by subtracting the amount deposited in the body from the amount absorbed. The quantity absorbed will be found in table 1. The amount deposited was obtained by subtracting 0.28 gm. from the total calcium in the body at the conclusion of the experiment and 0.18 gm. phosphorus from the total phosphorus as shown in table 2. These subtractions were made to allow for the amounts of these elements in the bodies of the animals at the beginning of the experiment. The values 0.28 and 0.18 gm. were obtained from analysis of animals at the age when the experimental feeding was begun. These rats were of the same weight and maintained under the same conditions as the experimental animals.

The calculations showed that the average calcium excretion in the urine over 70 days for the entire group of males and females was 0.46, 0.23 and 0.20 gm., respectively, on rations 1, 2 and 3 and the phosphorus excretion 0.79, 0.47 and 0.16 gm. These data are presented to show that with a decrease in the

TABLE 1

*Calcium and phosphorus absorption over a period of 70 days with different calcium and phosphorus intake*[1]

| RATION[2] | CALCIUM INTAKE | CALCIUM IN FECES | AMOUNT ABSORBED[3] | DECREASE IN ABSORPTION COMPARED WITH CONTROLS | PHOSPHORUS INTAKE | PHOSPHORUS IN FECES | AMOUNT ABSORBED[3] | DECREASE IN ABSORPTION COMPARED WITH CONTROLS |
|---|---|---|---|---|---|---|---|---|
| | | | | Males | | | | |
| | gm. | gm. | gm. | gm. | gm. | gm. | gm. | gm. |
| 1 | 8.82 | 5.77 | 3.05 | — | 5.05 | 2.72 | 2.33 | — |
| 2 | 5.84 | 3.37 | 2.47 | 0.58 | 3.42 | 1.58 | 1.84 | 0.49 |
| 3 | 3.46 | 1.18 | 2.28 | 0.77 | 2.06 | 0.65 | 1.41 | 0.92 |
| | | | | Females | | | | |
| 1 | 6.87 | 4.50 | 2.37 | — | 3.93 | 2.19 | 1.74 | — |
| 2 | 4.56 | 2.50 | 2.06 | 0.31 | 2.68 | 1.23 | 1.45 | 0.29 |
| 3 | 2.72 | 0.89 | 1.83 | 0.54 | 1.61 | 0.54 | 1.07 | 0.67 |
| | | | | Males and females | | | | |
| 1 | 7.84 | 5.14 | 2.70 | — | 4.49 | 2.46 | 2.03 | — |
| 2 | 5.20 | 2.94 | 2.26 | 0.44 | 3.05 | 1.41 | 1.64 | 0.39 |
| 3 | 3.09 | 1.04 | 2.05 | 0.65 | 1.84 | 0.60 | 1.24 | 0.79 |

[1] There were eight males and eight females on each ration; the values in the table are averages.

[2] Ration no. 1 contained the Osborne-Mendel salt mixture with slight modifications (see text); no. 2, approximately 67%, and no. 3, 41% as much calcium and phosphorus as ration no. 1.

[3] The difference between the intake and the amount recovered in the feces is regarded for convenience as the "amount absorbed."

TABLE 2

*Composition of rats with different calcium and phosphorus intake*[1]

| RATION | INITIAL WEIGHT | FINAL WEIGHT AFTER 24-HOUR FAST | INCREASE OVER INITIAL WEIGHT | WATER CONTENT | DRY WEIGHT | FAT | PROTEIN (N × 6.25) | CALCIUM | PHOSPHORUS | Ca/P RATIO |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | Males | | | | | |
| | gm. | gm. | % | % | gm. | gm. | gm. | gm. | gm. | |
| 1 | 34 | 259 | 662 | 57.8 | 109.3 | 44.6 | 47.8 | 2.72 | 1.56 | 1.7 |
| 2 | 33 | 254 | 670 | 56.7 | 110.0 | 48.3 | 44.8 | 2.50 | 1.48 | 1.7 |
| 3 | 34 | 257 | 656 | 57.8 | 108.5 | 46.7 | 44.8 | 2.32 | 1.42 | 1.6 |
| | | | | | Females | | | | | |
| 1 | 33 | 179 | 442 | 58.9 | 73.6 | 28.1 | 32.7 | 2.31 | 1.28 | 1.8 |
| 2 | 34 | 180 | 429 | 58.6 | 74.5 | 29.5 | 31.5 | 2.12 | 1.21 | 1.8 |
| 3 | 34 | 172 | 406 | 60.9 | 67.3 | 25.1 | 30.2 | 1.94 | 1.10 | 1.8 |

[1] Each value in the table is an average derived from eight experiments.

calcium and phosphorus intake there was a diminution in their output in the urine. It is worthy of note that a positive calcium and phosphorus balance occurred with the smallest intake.

*Water intake.* The averages of the water intake of the males and females on the three rations for the entire experiment were, respectively, 1160, 1163 and 1146 cc. It is of interest to note in this connection that on a diet containing approximately only 0.015% phosphorus, Day and McCollum (cited by McCollum, Orent-Keiles and Day, '39) found no marked increase in water consumption as is said to occur in phosphorus deficient animals.

*Body weight and body analysis.* The percentage gains in body weight and the absolute dry weights of the males were not significantly different on the three rations (table 2). The females, however, showed a significantly lower percentage gain on ration 3 than on ration 1. The dry weight of the females was also definitely smaller for the animals on ration 3 than for those on rations 1 and 2, whereas the dry weights of the males were the same on the three rations. While the percentage gains in weight and the dry substance of the bodies were smaller in the females on ration 3, the percentage water content was 2.0% and 2.3% higher than on rations 1 and 2, respectively. These higher percentages were definitely significant as P was equal to 0.013 and 0.005, respectively. The percentage water content in the males was not significantly different on the three rations.

The fat contents of the males were not significantly different on the three rations. The bodies of the females, however, contained a definitely smaller amount of fat on ration 3; but the difference on rations 1 and 2 was not significant.

The deposition of body protein in both the males and females was adversely affected by reducing the calcium and phosphorus intake. In the males the protein in the body was the same on rations 2 and 3 but in each instance definitely less than on ration 1. In the females the protein deposited was not significantly reduced on ration 2, but as shown in table 3, it was definitely less on ration 3.

TABLE 3

*Statistical analysis*

| | CALCIUM | | PHOSPHORUS | | DRY WEIGHT | | FAT | | PROTEIN | |
|---|---|---|---|---|---|---|---|---|---|---|
| | ♂ | ♀ | ♂ | ♀ | ♂ | ♀ | ♂ | ♀ | ♂ | ♀ |
| **Comparison of body constituents on rations 1 and 2** | | | | | | | | | | |
| Mean difference (gm.)[1] | +0.22 | +0.19 | +0.08 | +0.07 | −0.7 | −0.9 | −3.7 | −1.4 | +3.0 | +1.2 |
| Standard deviation | ±0.22 | ±0.17 | ±0.13 | ±0.12 | ±7.0 | ±4.0 | ±6.7 | ±5.2 | ±1.3 | ±1.96 |
| Probability | 0.016 | 0.012 | 0.072 | 0.094 | 0.233 | 0.30 | 0.09 | 0.253 | <0.001 | 0.075 |
| **Comparison of body constituents on rations 1 and 3** | | | | | | | | | | |
| Mean difference (gm.)[1] | +0.40 | +0.37 | +0.14 | +0.18 | +0.8 | +6.3 | −2.1 | +3.0 | +3.0 | +2.5 |
| Standard deviation | ±0.22 | ±0.25 | ±0.10 | ±0.11 | ±6.8 | ±2.9 | ±6.6 | ±3.1 | ±3.1 | ±2.1 |
| Probability | <0.001 | 0.004 | 0.003 | 0.002 | 0.33 | <0.001 | 0.214 | 0.018 | 0.019 | 0.008 |
| **Comparison of body constituents on rations 2 and 3** | | | | | | | | | | |
| Mean difference (gm.)[2] | +0.18 | +0.18 | +0.06 | +0.11 | +1.5 | +6.8 | +1.6 | +4.4 | 0 | +1.3 |
| Standard deviation | ±0.12 | ±0.15 | ±0.08 | ±0.05 | ±7.1 | ±5.1 | ±5.6 | ±3.1 | — | ±1.6 |
| Probability | 0.003 | 0.018 | 0.041 | <0.001 | 0.31 | 0.004 | 0.238 | 0.009 | — | 0.034 |

[1] + indicates a greater average content on ration 1; — the reverse.

[2] + indicates a greater average content on ration 2; — the reverse.

Correlatively with the decrease in absorption of calcium and phosphorus on rations 2 and 3 there was a smaller deposition of these elements in the body. The differences in the calcium content of the body on the various rations were all significant as shown in table 3. The calcium content of the bodies on the three rations was, respectively, 1.05, 0.98 and 0.90% of the body weight in the males and 1.29, 1.18 and 1.13% in the females. The percentage calcium in our control group was slightly higher than the normal values of 0.96% for males and 1.16% for females 90 days of age, as reported by Whitcher, Booher and Sherman ('36). The diet of their animals consisted of one-third whole milk powder, two-thirds ground wheat, and contained 0.83% calcium and 0.43% phosphorus. It will be observed that the calcium and phosphorus content was nearly the same as in our control ration. Our animals were 100 days old when sacrificed and heavier than those of Sherman and his co-workers. The difference in age and weight doubtless accounts for the difference in percentage calcium, because our values were approximately the same as for their older animals which were nearly equal in weight to our 90-day-old rats.

The phosphorus in the bodies of the animals on the three rations was, respectively, 0.60, 0.58 and 0.55% of the body weight for the males and 0.71, 0.67 and 0.64% for the females. Although the differences in the phosphorus content of the body were small, the differences between the animals on ration 3 as compared with those on rations 1 and 2 were significant. The difference, however, between rations 1 and 2 was not significant.

*Bone.* As shown in table 4, a progressive decrease in the calcium and phosphorus intake produced no change in the organic material of the femur but was accompanied by a progressive decline in the wet weight, dry weight, ash, calcium, and phosphorus content. Since the same order of differences was observed in the bones of the males and females on the different rations, the data on the two sexes were combined for statistical analysis. The lower values on ration 2 as com-

pared with those on ration 1 and on 3 as compared with ration 2 were definitely significant with one exception. The difference in the wet weight with respect to rations 2 and 3 was not significant as P was equal to 0.14. The lack of significance of the difference between these groups was doubtless due to the irregularity that occurred in the males, namely, a slightly heavier weight of the bone when on ration 3 as compared with 2. The difference of 26 mg. in the weight of the femur of the females on these two rations was definitely significant ($P = 0.02$).

TABLE 4

*Femur—weight and analytical data*

| RATION | WET WEIGHT | DRY WEIGHT | TOTAL ASH | ORGANIC MATTER | TOTAL CALCIUM | TOTAL PHOSPHORUS | Ca/P RATIO |
|---|---|---|---|---|---|---|---|
| | | | | **Males** | | | |
| | *mg.* | *mg.* | *mg.* | *mg.* | *mg.* | *mg.* | |
| 1 | 716 | 453 | 309 | 144 | 105.7 | 46.1 | 2.3 |
| 2 | 689 | 437 | 298 | 139 | 100.7 | 44.1 | 2.3 |
| 3 | 697 | 421 | 275 | 146 | 93.9 | 41.1 | 2.3 |
| | | | | **Females** | | | |
| 1 | 576 | 379 | 266 | 113 | 89.2 | 38.4 | 2.3 |
| 2 | 570 | 375 | 255 | 120 | 86.3 | 37.2 | 2.3 |
| 3 | 544 | 324 | 212 | 113 | 71.5 | 30.8 | 2.3 |
| | | | | **Males and females** | | | |
| 1 | 646 | 416 | 288 | 129 | 97.5 | 42.3 | 2.3 |
| 2 | 630 | 406 | 277 | 130 | 93.5 | 40.7 | 2.3 |
| 3 | 621 | 373 | 244 | 130 | 82.7 | 36.0 | 2.3 |

The values for calcium in the bone relative to the three rations and given in table 4 represented, respectively, 23.3, 23.0 and 22.3% of the dry weight of the bone in the males and 23.5, 23.0 and 22.1% in the females; those for phosphorus were 10.2, 10.1 and 9.8% in the males and 10.1, 9.9 and 9.5% in the females. The reduction of the calcium and phosphorus in the case of ration 2 as compared with ration 1 was extremely small, but it is worthy of note that the same results were obtained in the males and females. The total calcium and phosphorus in the bone in the case of ration 2 was 96% and

with ration 3, 85% of the amount present when the control ration was fed.

Although the total organic matter in the bone was not significantly different on the three rations, the percentage organic matter with reference to the dry weight showed a progressive increase with a reduction of the percentage calcium and phosphorus in the food mixture. On rations 1, 2 and 3 it was, respectively, for the entire group of males and females 31.0, 32.0 and 34.9%. The difference between rations 1 and 2 was probably significant ($P = 0.042$); the difference between rations 1 and 3 was definitely significant ($P = 0.0001$).

## DISCUSSION

The reduction in the weight of the bone that resulted from the smaller intake of calcium and phosphorus, and the change that occurred in the composition of the bone, namely, a lower percentage of calcium and phosphorus together with a higher percentage of organic material, appears to have been significant. It is possible that such a change weakened the bones. This question, however, should be put to an experimental test before definite conclusions are formulated.

While a lowering of the calcium-phosphorus intake in these experiments led to a decrease in the calcium, phosphorus, and protein deposition in the body, no conclusions can be drawn at the present time with regard to their practical import. Further investigation will be required to determine whether longevity, reproductivity, spontaneous activity, resistance to fatigue, endurance and the various physiological processes of the organism are adversely affected by the diminution in the calcium, phosphorus, and protein that occurred. No differences in the general condition of the animals on the different rations could be detected from gross observation.

A slight impairment in digestibility or absorption with the higher calcium and phosphorus intake is indicated by the larger amount of organic material in the feces. Since the animals did not gain more weight on a larger calcium and phosphorus intake notwithstanding a slightly greater

absorption of food material, it would appear that a slightly greater waste of energy as heat occurred in these animals than in those on the higher calcium and phosphorus intake.

In these experiments it was deemed advisable to maintain the Ca/P ratio constant; consequently, it is impossible to state whether the results obtained are to be attributed to the deficiency in the intake of calcium or of phosphorus or of both. To establish this point further work is needed in which the percentage calcium in the food mixture is reduced while the percentage phosphorus is maintained constant and vice versa, the normal ratio being disregarded.

## SUMMARY

The effects of lowering the calcium and phosphorus intake of albino rats were studied with respect to the calcium and phosphorus absorption over a period of 70 days and the various constituents of the body and the femur.

The basal ration contained 1% calcium and 0.57% phosphorus. Ration 2 contained approximately two-thirds and ration 3 less than one-half the amount of calcium and phosphorus in the basal ration. The Ca/P ratio was the same in the three rations.

On the lower calcium and phosphorus rations there was a progressive decrease in the amount of these elements in the feces. An appreciable amount of both calcium and phosphorus was nevertheless recovered on the ration giving the lowest intake.

Calculations indicate that over a period of 70 days on the three rations, 0.41, 0.23 and 0.20 gm. calcium and 0.79, 0.47 and 0.16 gm. phosphorus were excreted in the urine.

A decrease in the calcium and phosphorus content of the diet was accompanied by a slight increase in the amount of food material absorbed.

There was no significant difference in the water intake on the three rations. The animals were allowed to drink ad libitum.

The progressive decrease in the calcium and phosphorus percentage of the food mixture resulted in a corresponding diminution in the wet weight, dry weight, ash, calcium and phosphorus content of the bone and an increase in the percentage of organic material.

The calcium and phosphorus content of the body was correlatively less with the lower calcium and phosphorus intake.

Reduction in the calcium and phosphorus intakes had no effect on the body weight, dry weight or fat deposition except in the females on ration 3. In this case the gain in weight, the dry weight and fat content of the body were less than on rations 1 and 2.

The protein content of the body was less with the low calcium and phosphorus intake than on the basal ration.

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# THE RELATION OF PHYTIN TO THE CALCIFYING ACTION OF CITRATES[1]

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For several years citric acid and its salts have been occasionally recognized as possibly beneficial in the prevention of rickets in infants (Weissenberg, '28; Siwe, '38). Shohl ('37), Hamilton and Dewar ('37) and Hathaway and Meyer ('39), using the Steenbock-Black and McCollum ricketogenic diets, demonstrated that citrates have both a curative and preventive effect in rats when fed at high levels. A mixture of the free acid and the sodium or potassium salt was more antiricketic than either the acid or salts alone (Shohl, '37; Hathaway and Meyer, '39). That the beneficial effects are not attributable to acid-base factors is indicated by Shohl ('37) who found that among the organic acids tested only citrates and tartrates were effective. Hamilton and Dewar ('37), recognizing that citrates (and tartrates) form complexes with calcium, suggested that the antiricketic effect is possibly due to a decrease in available calcium, thus sparing phosphorus which is in relatively low concentration in the diets used. There is ample evidence that calcium forms a soluble complex with citrates in which the calcium ceases to be positively ionized (Starkenstein, '14; Shelling and Maslow, '28; Shear, Kramer and Resnikoff, '29; Greenberg and Greenberg, '32-33; Hastings et al., '34). Shohl and Butler ('39) have reported two cases of human rickets which they believe were benefited

[1] Aided by a grant from the Rockefeller Foundation.

by citrate administration. Eliot and Park ('37) concluded that, "Perhaps by means of sodium citrate it will be possible greatly to accelerate the cure of rickets and to treat successfully the forms of the disease which are refractory to all but enormous doses of vitamin D." Since rickets is characterized by inadequate mineralization of growing bone (Shohl, '39, p. 147) the relation of citrates to bone mineralization, in general, and rickets prevention and cure, in particular, should be further investigated.

Because the experimental diets used by previous investigators contained a large percentage of corn, known to contain variable amounts of biologically unavailable phosphorus (Harris and Bunker, '35), it seemed important to determine the calcifying effect of citrates on bones, using a purified diet containing different amounts of calcium and phosphorus of high biological availability. In case the beneficial effect on ossification could not be produced with purified diets, but only with corn diets containing an excess of calcium carbonate, it would seem possible that the effect might be related to the phytin of the unpurified diets. Lowe and Steenbock ('36a) found that, "Phytin-P is not completely unavailable to the rat, and secondly that the small amount which might be available is rendered almost completely unavailable by the presence of $CaCO_3$." It would be a plausible assumption, therefore, that citrates added to diets containing phytin and an excess of calcium carbonate reduce the ricketogenic (anticalcifying) action of such diets by forming a citrate complex with the calcium, thus permitting more of the phytin phosphorus to be hydrolyzed and utilized.

## EXPERIMENTAL

Young rats weighing 45 to 60 gm. were used. The sexes and litters were equally distributed in each experiment. Three or four rats were kept in each cage. Food was given ad libitum.

At the end of each experiment, 20 or 21 days, the animals were killed with ether and the left hind leg of each was removed. Soft tissue was loosened by boiling 3 minutes in

distilled water. The bones were carefully cleaned and the femur, tibia and fibula of each leg were extracted 48 hours with ethyl alcohol and 48 hours with ethyl ether. After drying 24 hours at 102°C., they were placed in a cold muffle furnace and ashed 14 to 18 hours at about 550°C. Bone ash (total ash and percent ash) determination was relied upon to appraise the calcifying effectiveness of citrates in the various diets because it is entirely objective, and in a procedure as was employed here, the method is more accurate than the alternative histological or roentgenological technics (Coward, '38). Lowe and Steenbock ('36b) depended exclusively upon bone analysis (total ash and percent ash) in studies on the rickets-ogenic properties of corn. Such a procedure is essential if the antiricketic effectiveness of a substance is to be measured in terms with practical meaning, i.e, the degree to which mineralization of bone is facilitated.

The composition of all basal diets used is given in table 1.

TABLE 1

*Basal diets*

| CONSTITUENTS | DIETS | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 |
| | % | % | % | % | % | % | % | % |
| Yellow corn | 73 | 73 | 73 | | | | | |
| Wheat gluten | 20 | 20 | 20 | | | | | |
| NaCl | 1 | 1 | 1 | | | | | |
| Casein, acid washed | | | | 15 | 15 | 15 | 15 | 15 |
| Edestin | | | | 3 | 3 | 3 | 3 | 3 |
| Dextrin | | | | 20 | 20 | 20 | 20 | 20 |
| Sucrose | 3 | 2.5 | 2 | 40.7 | 40.4 | 39.9 | 39.2 | 39.4 |
| Yeast, bakers' | | | | 5 | 5 | 5 | 5 | 5 |
| Salts no. 4 (Ca-P free) | | | | 3 | 3 | 3 | 3 | 3 |
| Hydrogenated fat (Crisco) | | | | 10 | 10 | 10 | 10 | 10 |
| Carotene in corn oil (3:1000) | | | | 0.3 | 0.3 | 0.3 | 0.3 | 0.3 |
| $CaCO_3$ | 3.0 | 1.5 | 0.5 | 3.0 | 1.5 | 0.5 | 3.0 | 1.0 |
| $NaH_2PO_4 \cdot H_2O$ | | 2.0 | 3.5 | | 1.8 | 3.3 | | 1.8 |
| Phytin | | | | | | | 1.5 | 1.5 |

The edestin was of "technical" grade.[2] The percentage composition of salts no. 4 was as follows: $NaHCO_3$, 37.21; $MgSO_4$, 10.14; KCl, 48.53; $FeCl_3 \cdot 6\ H_2O$, 3.21; $CuSO_4 \cdot 5\ H_2O$, 0.44; $MnSO_4 \cdot 2\ H_2O$, 0.14; $ZnSO_4 \cdot 7\ H_2O$, 0.30 and KI, 0.02. Phytin, containing 14.2% phosphorus, was prepared from wheat bran, according to Boutwell's ('17) procedure. It was free from inorganic phosphorus.

Eight basal diets were used (table 1). In all experimental diets citrates were added by replacement of equal weights of sucrose. Diets 1a-3a (table 2) contained 2% citrate mixture (citric acid 19.2 gm., sodium citrate 28.6 gm.). Diets 4a-8a (table 2) contained 4% of the same mixture. The citrate mixtures, fed at these levels, caused no appreciable effect on the food consumption.

## RESULTS

Table 2 summarizes the data. In order to conserve space only mean values are presented, with calculations of the statistical significance of the mean differences expressed as $t$ (Snedecor, '38, p. 57).

It is shown that a citric acid-sodium citrate mixture at the moderately low level of 2% permits improved ossification when fed with the high calcium-low phosphorus Steenbock-Black ricketogenic diet (diets 1 and 1a). The content of bone ash in the citrate fed rats possibly was raised significantly ($t = 1.62$). However, $t$ would have to be 2.30, or higher, to be unequivocally significant. With a moderate amount of calcium and phosphorus (diets 2 and 2a) the percent bone ash was even lower in the citrate-fed rats than in the controls, but the difference may not be significant ($t = 1.95$). The presence of citrate in the basal diet made rich in phosphorus and poor in calcium (diets 3 and 3a) was without effect on the total ash and percent ash. It may be significant, however, that all of the rats fed citrate lost weight while one-half (four) of the controls made small gains in weight, although the average for all the controls showed a slight loss of weight.

[2] Obtained from the Arthur H. Thomas Company.

TABLE 2

*Calcifying action of a citric acid-sodium citrate mixture in diets containing (1) corn, (2) purified components and (3) purified components plus phytin*

| DIET | | | CITRATE MIXTURE | NO. OF RATS | WEIGHT INCREASE | BONE ASH | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | Mg. | | | per cent | | |
| No. | Ca | P | | | | Mean | Deviation from mean | t | Mean | Deviation from mean | t |
| **Experiment 1 (corn)** | | | | | | | | | | | |
| | % | % | % | | gm. | | | | | | |
| 1 | 1.25 | 0.25 | — | 7 | 18 | 54.3 | 8.2 | | 37.7 | 1.8 | |
| 1a | 1.25 | 0.25 | 2 | 7 | 19 | 64.8 | 8.7 | 1.62 | 42.3 | 1.7 | 3.61 |
| 2 | 0.65 | 0.75 | — | 8 | 7 | 84.3 | 8.4 | | 53.2 | 1.2 | |
| 2a | 0.65 | 0.75 | 2 | 8 | 3 | 82.3 | 11.4 | 0.29 | 51.7 | 1.1 | 1.95 |
| 3 | 0.25 | 1.05 | — | 8 | −1 | 65.9 | 6.6 | | 46.7 | 1.5 | |
| 3a | 0.25 | 1.05 | 2 | 8 | −6 | 68.1 | 6.6 | 0.54 | 47.1 | 1.0 | 0.49 |
| **Experiment 2 (purified components)** | | | | | | | | | | | |
| 4 | 1.25 | 0.20 | — | 7 | 32 | 77.9 | 9.3 | | 43.5 | 1.3 | |
| 4a | 1.25 | 0.20 | 4 | 7 | 28 | 81.3 | 13.8 | 0.44 | 44.8 | 1.7 | 1.50 |
| 5 | 0.65 | 0.65 | — | 7 | 24 | 103.7 | 9.7 | | 54.9 | 0.8 | |
| 5a | 0.65 | 0.65 | 4 | 7 | 20 | 95.3 | 8.6 | 1.51 | 53.4 | 1.1 | 2.31 |
| 6 | 0.25 | 0.95 | — | 8 | 4 | 66.6 | 3.6 | | 50.1 | 0.6 | |
| 6a | 0.25 | 0.95 | 4 | 8 | 6 | 67.2 | 4.7 | 0.23 | 50.3 | 1.7 | 0.25 |
| **Experiment 3 (purified components plus phytin)** | | | | | | | | | | | |
| 7 | 1.25 | 0.40 | — | 8 | 35 | 90.8 | 8.9 | | 43.8 | 1.7 | |
| 7a | 1.25 | 0.40 | 4 | 8 | 36 | 110.3 | 11.8 | 2.87 | 48.1 | 0.9 | 5.00 |
| 8 | 0.45 | 0.80 | — | 8 | 20 | 103.1 | 14.3 | | 50.6 | 1.0 | |
| 8a | 0.45 | 0.80 | 4 | 8 | 20 | 102.7 | 12.0 | 0.04 | 51.9 | 1.2 | 1.92 |

The favorable effect of citrates observed with diet 1a did not occur when a high calcium-low phosphorus purified diet was fed (diets 4 and 4a), even though it contained twice as much citrate. Also, there was no effect with moderate calcium-moderate phosphorus (5 and 5a) or low calcium-high phosphorus diets (6 and 6a). It should be noted that the total bone ash of rats fed the high calcium-low phosphorus corn diets (1 and 1a) is definitely lower than that of rats fed the high calcium-low phosphorus purified diets (4 and 4a). Likewise, the former rats gained less weight than the latter. By all the criteria employed, rats receiving the corn diets developed less favorably than animals fed the corresponding purified diets containing approximately the same content of calcium but less phosphorus. In planning these rations no attempt was made to render the purified diets equivalent to the corn diets with respect to total phosphorus content owing to the variable biological availability of phosphorus in the latter.

On the basis of results from these experiments it appeared evident that citrate in purified diets was without significant effect on ossification whatever the levels of calcium and phosphorus, and in rations composed principally of corn the beneficial effects on bones occurred only when there was an excess of calcium carbonate. Hence it was of interest to observe the effect of adding the principal phosphorus compound of corn, phytin, to purified diets containing citrate. There is no doubt that citrate was beneficial in the presence of an excess of calcium (diets 7 and 7a). Both the total bone ash and the per cent ash were significantly increased. But with moderate levels of calcium and phosphorus (diets 8 and 8a) neither the total ash nor the per cent of ash were affected by the presence of citrate.

## DISCUSSION

The data reported here are in general conformity with the findings of previous investigators as respects the beneficial effects of citrates on ossification in rats fed a high calcium-low phosphorus cereal ration.

However, the citrate mixtures were fed at a much lower level than in the experiments of Shohl ('37), Hamilton and Dewar ('37) and Hathaway and Meyer ('39). Their diets contained as much as 20% or more citrate. But apparently they experienced little difficulty in getting the animals to eat the diets since Hathaway and Meyer ('39), who reported food consumption data, noted no appreciable depression of weight increase or food consumption. Possibly higher levels of citrate in our diets would have produced more exaggerated changes in the bones affected but it does not seem probable, in view of the indicated effect on phytin, that the non-significant values would have become significant.

With due regard for differences in experimental technic, our findings appear to be at variance with those of Shohl ('37), with respect to the effect of citrates on rats fed a low calcium-high phosphorus cereal diet. Shohl depended principally upon histological evidence while in the studies reported here the criteria used were the total bone ash and the per cent ash (from fat-free bones).

It is possible that Shohl's animals did not respond to citrate by an increase in ossification since the effect on total bone ash is not given. Indeed, as pointed out by Hathaway and Meyer ('39), "His (Shohl) table 2 shows that with diets S and K, slightly basic basal diets, the addition of the citric acid plus sodium citrate cured the rickets (by histological and x-ray diagnosis) but materially lowered the bone ash values, in diet S by 4%, in diet K by 9%." It is possible that our citrate fed rats (table 2, diet 3a) did not develop rickets, on the basis of histological criteria, although the failure of ossification was as great as in the control animals not fed citrate. Therefore, it is possible that there is no actual variance between Shohl's findings and those reported here since he was dealing with rickets only, and our data are confined to evidence on the mineralization of bone. Hathaway and Meyer ('39) have commented adequately on the relative merits of these technics, as respects the appraisal of calcifying agents, pointing out that, "improvement in bone ash indicates improved calcification

throughout the bone and 'cure of rickets' with continuance of osteoporosis does not materially improve the condition of the animals." Chick, Korenchevsky and Roscoe ('26) have shown that chemical analyses of bones furnish a better criterion of defective calcification than histological examination alone.

Hamilton and Dewar ('37) suggested that, "The beneficial effect of citrate and tartrate is probably exerted largely in the intestinal tract and is possibly due to a decrease in available calcium." The inapplicability of this suggested explanation is shown by the data reported here since citrate was without effect in high calcium-low phosphorus diets that did not contain phytin, either as such or in corn. If the effect was simply a matter of preventing phosphate precipitation in the intestinal tract, through binding of the excess calcium, calcification should have been improved in our high calcium-low phosphorus purified diet.

It seems probable that the explanation must be sought in the effect of citrates, directly or indirectly, on the biological availability of phosphorus complexes in the basal diets, as indicated previously. The findings of Lowe and Steenbock ('36a) would seem to be extremely pertinent in this respect since they clearly demonstrated that phytin phosphorus is rendered less available in the intestinal tracts of rats by the presence of excess calcium carbonate. It is a plausible assumption, therefore, that the beneficial effect of citrates is mediated through the formation of a calcium complex which interferes less, if at all, with the utilization of phosphorus in phytin, and perhaps other complexes of relatively low biological availability. That the effect is not simply one of producing a more favorable reaction (pH) for phytase activity is not very probable since, as observed by Shohl ('37), some other organic acids such as acetic, lactic, malic, malonic and succinic, and their sodium salts, do not improve the condition of the bones.

In view of the evidence reported here it would seem that there is need for caution in applying to problems of human

rickets the findings based on rickets prevention in animals fed experimental high calcium-low phosphorus cereal rations containing citrate. If the principal effect of citrates on calcification is as postulated here, there seems to be scarcely any basis in fact for the opinion that citrate administration might constitute an effective means of preventing human rickets, or of exerting any significant antiricketic effect. This is a plausible conclusion since the diets of infants and children, although generally containing considerable quantities of phytin, are not characterized by excesses of calcium. The fact cannot be ignored, however, that Shohl and Butler ('39) have concluded, on the basis of preliminary results from two cases, that a citric acid-sodium citrate mixture "not only offers an adjunct to accepted measures for the treatment of infantile rickets, but also may have application to other types of disorders of calcium and phosphorus metabolism." Obviously this is an insufficient number of cases to warrant reliance on the findings, particularly since complicating factors intervened as Shohl and Butler have recognized. It is necessary to mention that Albright et al. ('40), in a report on one case of "nephrocalcinosis with rickets and dwarfism", conclude that administration of a citric acid-sodium citrate mixture was of benefit in treatment of the disease. However, in this instance the primary metabolic disturbance appeared to be an acidosis which resulted in loss of base, including calcium. This eventuated in 'low phosphorus' rickets. Hence, as Albright et al. have suggested, the benefit probably derived from the sodium and not the citrate.

Chaney and Blunt ('25) observed a definite increase in calcium and phosphorus retention of girls fed large amounts of orange juice. This has been cited as possible evidence of the favorable effect of citrates on calcification (Hamilton and Dewar, '37; Shohl and Butler, '39). It should be recognized, however, that the control diet was deficient in ascorbic acid and possibly other essential nutrients. Since marked nitrogen retention occurred also, when orange juice was fed, it is at least equally plausible that the favorable mineral retention is

attributable to the ascorbic acid ingestion. However, the finding of Lanford ('39), that rats on a whole wheat-milk powder diet retain more calcium when fed orange juice, is not so readily explained on the basis of ascorbic acid supplementation, because the rat, presumably, is not benefited by dietary ascorbic acid. Since the Ca:P ratio of Lanford's diet was not excessive, her results do not appear explicable on the same basis as the data reported here. Whether citrate alone would yield the same effect with Lanford's diet must be determined before it can be concluded that her findings and those reported here are contradictory.

The data presented in this paper, and the discussion of other findings, emphasize again the difficulties that may arise in the exclusive use of cereal rations in the study of rickets and other problems concerned with calcium and phosphorus utilization. Further investigation is needed to reveal the mechanism whereby citrate promotes better ossification when the diet contains phytin and an excess of calcium.

## SUMMARY

Studies have been made of the effect on bone ash of young rats fed (a) cereal rations and (b) "purified" rations containing a mixture of citric acid and sodium citrate and different amounts of calcium and phosphorus.

The finding of other investigators has been confirmed that citrates promote calcification in the bones when added to cereal rations high in calcium and low in phosphorus.

With a purified ration citrate does not promote calcification when the calcium level is high and phosphorus is low.

Citrates have no appreciable effect on calcification when the diet contains moderate levels of calcium and phosphorus, or is low in calcium and high in phosphorus, irrespective of whether a cereal ration or a purified one is used.

Citrates added to a purified ration containing phytin cause a definite increase in calcification if the Ca:P ratio is high, but there is no effect with a moderate Ca:P ratio.

It is indicated that a beneficial effect of citrates in ricketogenic diets does not occur unless there is a high Ca:P ratio and appreciable quantities of phytin, or perhaps other phosphorus-containing complexes of low biological availability.

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# A BIOLOGICAL ASSAY OF RIBOFLAVIN IN THE LIVER OF THE COW, CALF, SHEEP, LAMB, AND HOG [1]

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TWO FIGURES

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Riboflavin is found rather abundantly in a variety of foodstuffs including milk, lean meat, liver, and the green growing parts of plants. Since information is lacking upon the riboflavin content of other kinds of liver than beef and pork (Daniel and Munsell, '37; Hoagland and Snider, '30), this study was undertaken to obtain additional information regarding the riboflavin values of livers from various animals.

## PROCEDURE

Livers for these experiments were purchased in August, September, October, and again in December, 1936, at local retail markets which had obtained their meats from packing plants in Kansas City. In the fall one sample each was purchased of beef, calf, pork, and lamb livers. Each sample represented two to six animals. In December, a second set of samples was purchased and sheep liver was included.

The thick blood vessel walls in the liver and any inedible portions that would ordinarily be discarded if the liver were to be eaten were removed and the raw livers run through a food chopper having a fine blade; after mixing they were put through the chopper a second time. They were then placed in glass containers and stored at a temperature below 0°C.

[1] Contribution no. 88, Department of Home Economics.

Small amounts were removed as needed for feeding experiments.

The Bourquin-Sherman ('31) method was used and the tests run in conjunction with riboflavin [2] as a standard. Booher, Blodgett and Page ('34) and also Bisbey and Sherman ('35) and Bessey ('38) have shown this method of assay to be suitable for quantitative determinations of riboflavin in foods.

The casein was prepared according to the method suggested by Ellis ('32). The Osborne and Mendel salt mixture was made by the time-saving method suggested by Wesson ('32), and the butterfat was melted and filtered at 45° to 55°C.

One hundred and thirty-six albino rats of The Wistar Institute strain, raised on the diet described by Sherman and Crocker ('22), were used in this experiment. They were placed on the riboflavin-free diet at 3 weeks of age if they reached an approximate weight of 38 to 40 gm. In our laboratory rats 3 weeks old have been found to be more satisfactory than 28-day old animals. This is in accord with the work of Lassen ('36). Toward the end of the depletion period the rats were weighed daily, and when the weights were judged to be stationary, the animals were put in individual cages and supplements of test material allotted. The average depletion period was 14 days and the average weight at the time of depletion was 48 gm. with a range of 40 to 56 gm.

The group of animals on each sample of liver generally included ten or more individuals, and was made up approximately of the same number of males and females. Nine groups received the test livers, two received riboflavin, and one received only the basal diet and water.

The animals receiving liver were given 200 mg. twice weekly. They liked the liver and consumed it within a few seconds so that there was no difficulty in getting quantitative consumption of the test food. A rat from each litter used in the experiment was given 2.5 or 5.0 mcg. of riboflavin and one was given no supplement. The riboflavin solution was made up to 1 mg. per 100 ml. of distilled water and was fed to the rats at two levels,

[2] "Lactoflavin, PX grade" from the Borden Company was used.

namely 2.5 and 5.0 mcg., daily. The solution was dropped directly into the mouths of the animals from a 1-ml. syringe graduated in hundredths.

During the test period, the animals were weighed each week and records were made of the amount of food consumed.

TABLE 1

*Riboflavin content of liver from various animal sources collected during the fall and winter seasons, as revealed by rat feeding experiments*

| SUPPLEMENT FED | DAILY PORTION FED | NUMBER RATS | AVERAGE DEPLETION PERIOD | AVERAGE INITIAL WEIGHT | AVERAGE GAIN IN WEIGHT | ESTIMATED RIBOFLAVIN PER 100 GM. |
|---|---|---|---|---|---|---|
| | *mg.* | | *days* | *gm.* | *gm.* | *micrograms* |
| Lamb liver | | | | | | |
| fall | 66.7 | 15 | 14 | 45 | 22 | 4950 |
| winter | 66.7 | 10 | 13 | 47 | 24 | 5400 |
| Mutton liver | | | | | | |
| winter | 66.7 | 10 | 16 | 49 | 20 | 4350 |
| Calf liver | | | | | | |
| fall | 66.7 | 10 | 14 | 46 | 15 | 3450 |
| winter | 66.7 | 12 | 16 | 50 | 20 | 4350 |
| Beef liver | | | | | | |
| fall | 66.7 | 12 | 14 | 46 | 13 | 2850 |
| winter | 66.7 | 8 | 14 | 47 | 16 | 3450 |
| Pork liver | | | | | | |
| fall | 66.7 | 15 | 14 | 47 | 12 | 2700 |
| winter | 66.7 | 11 | 15 | 48 | 12 | 2700 |
| None | 0 | 16 | 15 | 50 | −1 | |
| | *micrograms* | | | | | |
| Riboflavin | 2.5 | 10 | 13 | 46 | 17 | |
| Riboflavin | 5.0 | 9 | 14 | 49 | 33 | |

Observations were made upon the activity and appearance of an animal at the end of the experimental period and any unusual behavior during the test period was recorded. The record of any animal suspected of coprophagy was not used in the final summary. This accounts for the smaller number of animals in some groups.

## FINDINGS

A summary of the data from the feeding experiments is given in table 1. At the end of the 8-week period, the test ani-

mals showed a gain of 12 to 24 gm. of weight. The negative control animals lost 1 gm. of weight. The control group given 2.5 mcg. riboflavin per day showed an average gain of 17 gm., and the group fed 5.0 mcg., an average gain of 33 gm. The gains of these groups compared favorably with those of Ansbacher's (Ansbacher, Supplee and Bender, '36) experimental animals.

The positive control groups and the test animals fed livers containing highest riboflavin content had bright eyes, glossy

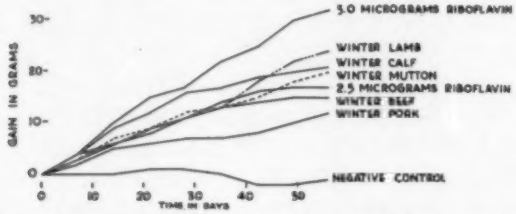


Fig. 1 Growth curves of rats on riboflavin-free diet plus animal livers collected during the winter season compared with 0.0, 2.5, and 5.0 mcg. of riboflavin fed daily to the control groups.

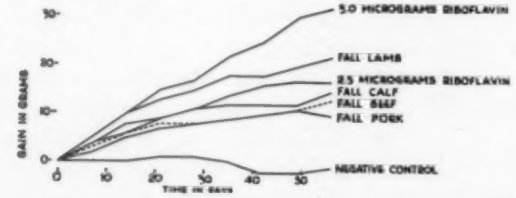


Fig. 2 Growth curves of rats on riboflavin-free diet plus animal livers collected during the fall season compared with 0.0, 2.5, and 5.0 mcg. of riboflavin fed daily to the control groups.

fur, good muscular tone, and a normal appetite. They were vigorous in their activity and seemed to be satisfied. The test animals fed pork liver as a source of riboflavin were vicious, restless, and lighter in weight, with shaggy fur and dull eyes. The negative control group lacked appetite and good muscular tone, and were light in weight; their general appearance was extremely poor, as the fur was rough, the eyes listless, the posture hump-backed, and movements sluggish.

The lamb liver showed the greatest potentiality for improved growth in rats while mutton was second; calf, third; beef, fourth; and pork, lowest as shown in table 1 and figures 1 and 2. The riboflavin values for the different samples were estimated by interpolating between the gains in weights by the groups of rats on 2.5 and 5.0 mcg. of riboflavin. Where the groups of animals on liver showed weight gains less than either one of the groups of riboflavin, values were estimated by extrapolation on the basis of an assumed curve of response.

## SUMMARY AND CONCLUSIONS

1. Samples of beef, calf, lamb, and pork livers were purchased in the early fall, then again in early winter with the addition of mutton livers. Each of the five liver samples represented two to six animals.

2. The assay for riboflavin was made according to the procedure of Bourquin and Sherman. Riboflavin fed at 0.0, 2.5, and 5.0 mcg. levels daily was used as a standard. The riboflavin content of the livers was estimated by comparing the gain in weights of the rats given the liver supplements with those given the riboflavin supplement.

3. Lamb liver, which showed the greatest potentiality for gain in weight in rats, was estimated to contain 4950–5400 mcg. per 100 gm.; mutton liver, 4350 mcg.; calf liver, 3450–4350 mcg., and beef liver, 2850–3450 mcg. per 100 gm. Pork liver, estimated to contain 2700 mcg. per 100 gm., ranked lowest in riboflavin value.

4. Liver samples purchased in the winter season were slightly higher in riboflavin value than samples purchased in the fall.

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# THE RIBOFLAVIN CONTENT OF CERTAIN HAYS AND GRASSES [1]

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(Received for publication March 20, 1940)

Since riboflavin is part of the vitamin B complex and is necessary for the growth and normal functioning of animals, a further interest has developed in studying its natural sources and the conditions under which it is preserved or destroyed in natural products.

Hays and grasses are used, more or less, in the feeding of every type of farm animal. Poultry especially require a liberal quantity of green, leafy hay or meal in order to maintain a high level of production and hatchability. Because of the extensive use of alfalfa leaf meal in poultry rations to supply the riboflavin, it was thought advisable to make a study of the influence of time of cutting and method of curing on the riboflavin content of some common hay plants and some grasses not commonly used commercially.

## EXPERIMENTAL PROCEDURE

### *The plants*

The description of the plants, time of cutting, and method of curing are given in table 2. Those plants cured in the sun were exposed on a western slope between the hours of 9:00 A.M. and 4:00 P.M. for a total of 48 hours. This is much longer than hay is ordinarily cured, but it was thought desirable to give the samples extreme exposure in order to note the effect of sunlight on the riboflavin content. Neither dew nor rain was

[1] Published with the permission of the Director of the Ohio Agricultural Experiment Station, Wooster.

allowed to fall on the samples. Practically all of the green color was destroyed during this exposure. After drying, the hays were ground to a very fine powder in a ball mill and stored at refrigeration temperature until time for assay.

### *Biological assay*

The rat growth method was used for the assay of the samples for riboflavin. The rats were confined in screen-bottom wire cages when weighing 35 to 45 gm. and fed the basal ration. The basal ration, consisting of vitamin-free casein 20 parts, sucrose 71, salt mixture 4, Crisco 3, and cod

TABLE 1

*Growth rate of rats. Weight increase on varying amounts of riboflavin; average of four experiments*

| SUPPLEMENT | NUMBER OF ANIMALS | | AMOUNT FED DAILY | AVERAGE INCREASE IN WEIGHT 6 WEEKS | AVERAGE INCREASE IN WEIGHT OVER NEGATIVE CONTROLS |
|---|---|---|---|---|---|
| | ♂ | ♀ | *micrograms* | *gm.* | *gm.* |
| Positive control, synthetic riboflavin (Merck) | 16 | 16 | 2.5 | 32 | 25 |
| | 16 | 16 | 5.0 | 55 | 48 |
| | 16 | 16 | 10.0 | 82 | 75 |
| | 16 | 16 | 20.0 | 106 | 99 |
| | 16 | 16 | 40.0 | 121 | 114 |
| Negative controls | 12 | 12 | 0.0 | 7 | — |

liver oil 2 parts respectively, was supplemented with daily addenda of 10 mcg. of thiamin [2] and a water-soluble fraction of yeast from which the greater part of the riboflavin had been removed by treatment with fuller's earth (see negative controls tables 1 and 2). The amount of this yeast extract fed daily was equivalent to 1.5 gm. dried yeast. This same amount of yeast extract when supplemented with adequate amounts of riboflavin and thiamin produced excellent growth, i.e., 18 to 20 gm. per week, and the same yeast extract, in amount of 0.25 cc. (0.25 gm. yeast), also cured acrodermatitis of rats.

[2] From the Merck Company, Rahway, New Jersey.

When the rats had ceased to gain in weight they were transferred to individual cages and the finely ground samples of the test hay or grass were fed, in separate dishes, as the source of riboflavin. The supplemental feeding was continued for 6 weeks.

The curve obtained by plotting the weight increment due to the feeding of different levels of pure riboflavin (minus the

TABLE 2

*Riboflavin content of hays and grasses*

| FEED SUPPLEMENTS | TIME OF CUTTING | METHOD OF CURING | NUMBER OF ANIMALS | | AMOUNT FED DAILY | AVERAGE INCREASE IN WEIGHT 6 WEEKS | AVERAGE INCREASE IN WEIGHT OVER NEGATIVE CONTROLS | ESTIMATED RIBOFLAVIN CONTENT PER GRAM |
|---|---|---|---|---|---|---|---|---|
| | | | ♂ | ♀ | *mg.* | *gm.* | *gm.* | *micrograms* |
| Oat plant | June 4: 8 to 10 in. high; before jointing | Subdued light | 4 | 4 | 75 | 32 | 20 | 26 |
| Wheat plant | May 21: 10 to 12 in. high; jointing | Subdued light | 4 | 4 | 75 | 27 | 15 | 17 |
| Bluegrass | May 21: beginning to head | Subdued light | 4 | 4 | 200 | 32 | 20 | 10 |
| Alfalfa | May 21: before bloom | Subdued light | 4 | 4 | 200 | 34 | 32 | 16 |
| Alfalfa | May 21: before bloom | 48 hours strong sunshine | 4 | 4 | 200 | 37 | 25 | 12 |
| Alfalfa | June 7: beginning to bloom | Subdued light | 4 | 4 | 300 | 48 | 36 | 12 |
| Alfalfa | June 7: beginning to bloom | 48 hours strong sunshine | 4 | 4 | 300 | 40 | 28 | 9 |
| Timothy | June 1: before heading | Subdued light | 4 | 4 | 300 | 61 | 49 | 17 |
| Timothy | June 10: in head before blooming | Subdued light | 4 | 4 | 300 | 52 | 40 | 12 |
| Negative control | | | 4 | 4 | | 12 | | |

negative controls) was used to calculate the riboflavin content of the products under study. This method is somewhat similar to that of Supplee and associates ('39).

## RESULTS AND DISCUSSION

The results of the assays of the different samples are given in table 2. The differences in the riboflavin content of the oat, wheat, and bluegrass plants, as noted in table 2, may be accounted for by a difference in the maturity of the plants, as shown under the heading, "Time of cutting," and more completely in a previous publication (Hunt, Record and Bethke, '36). There is no evidence in this study and in the previous one that any one plant of this type is superior to another as a source of riboflavin. The data do show that plants of this type are a very good source of riboflavin and offer possibilities as a natural source of riboflavin for practical purposes.

The assays of the alfalfa plant show that the riboflavin content per unit of weight decreased as the plant matured. There was a loss of about 25% due to a difference of about 2 to 3 weeks in maturity. The results from the alfalfa cut May 21 and June 7 are in close agreement with the data presented by Heiman and associates ('38) in which they compared the vitamin G (riboflavin) content of alfalfa hay cut when 10 and 24 inches high. The riboflavin content of the alfalfa cut May 21 is also in agreement with that found by Norris and associates ('36) who obtained a value of 16 chick units of vitamin G per gram of dehydrated alfalfa leaf meal (a chick unit is equivalent to 1 mcg. of riboflavin). Jukes ('37) obtained a value of 8 rat units of vitamin G per gram for dehydrated alfalfa meal. Hunt, Record and Bethke ('35, '36) obtained values of 6 to 12 units of vitamin G per gram of alfalfa hay. Data from this laboratory (unpublished) show that 1 rat unit (Bourquin-Sherman) is equivalent to 2 mcg. of riboflavin. Applying these data would give a value of 16 mcg. for Juke's sample and 12 to 24 mcg. for those obtained from this laboratory, whereas Supplee and associates ('39) obtained a value of 28 mcg. for

alfalfa meal. The apparent difference in the results obtained in these experiments and those obtained in a previous one is probably due to a difference in the maturity of the plants used or the percentage of leaf content in the sample.

The results obtained from alfalfa hays exposed to bright sunshine for 48 hours show that there is a loss of about 25% of their riboflavin content. That there was no greater loss may mean that part of the riboflavin may exist in these plants in the combined form. The data show, however, that hays used as a source of riboflavin should be exposed to sunshine for as short a time as is consistent with good practice.

The riboflavin content per unit of weight of the timothy plant also decreased as the plant matured. This loss is about 33% for a difference of about 10 days in maturity. The assays show that young timothy plants, as well as oats, wheat, and bluegrass, may contain as high a riboflavin content as early cut alfalfa.

The data shown in table 2 suggest that hays intended to supply the riboflavin requirements of animals should be cut before or not later than time of blooming and that the product should not be exposed to strong sunshine any longer than is necessary for proper curing. Curing in windrows or cocks, as is now done in good practice, will accomplish this.

## SUMMARY AND CONCLUSION

The riboflavin content, per unit of weight, of the alfalfa and timothy plants used in this study decreased as the plants matured. Oat, wheat, and bluegrass plants were studied only at one stage of maturity.

Alfalfa plants exposed to strong sunshine for 48 hours after cutting suffered a loss of about 25% of their riboflavin content.

Plants such as oats, wheat, bluegrass, and timothy offer a very good source of riboflavin in the early stages of development, and a more extended use of such products as a practical source of riboflavin is suggested.

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# HISTOLOGICAL STUDIES OF THE TISSUES OF RATS FED A DIET EXTREMELY LOW IN PHOSPHORUS [1]

RICHARD H. FOLLIS, JR., HARRY G. DAY AND E. V. McCOLLUM

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ONE PLATE (SIX FIGURES)

(Received for publication April 22, 1940)

Although an extensive literature has grown up dealing with the histological alterations in the skeletal system of rats in which high calcium-low phosphorus rickets had been produced, the other tissues of such animals for the most part have been neglected. Furthermore, no appreciable attention has been given to the microscopic study of tissues from animals on diets low in phosphorus but containing a normal amount of calcium, vitamin D and other nutrients. Therefore, it is of interest to examine the tissues of animals on a diet extremely deficient in phosphorus in order to determine whether or not extra-skeletal histo-pathological changes can be detected.

## EXPERIMENTAL

### *Material and methods*

The basal experimental diet used in these studies was reported by Day and McCollum ('39). Its percentage composition was as follows: edestin 16.0, gelatin 4.0, sucrose 61.2, salts (P-free) 4.0, choline hydrochloride in sucrose (1:9) 1.0, cystine in sucrose (1:19) 1.0, hydrogenated fat [2] 10.0, carotene in corn oil (3:1000) 0.3, viosterol 15 drops (3800 I. U.) per

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kilogram, and vitamin B complex 2.5%. This diet contained 0.017% phosphorus and 0.4% calcium and was designated diet 16 by Day and McCollum ('39); when supplemented with phosphorus (0.27%), it became diet 17.

A total of seventy-three rats were used. They were placed on the diets when they averaged about 50 gm. in weight. Two consecutive series were studied. Series I consisted of fifty-seven animals about equally distributed as to sex. They were fed ad libitum. Twenty-eight animals received diet 16, while twenty-nine received diet 17. Six animals (three experimental and three controls) were killed at weekly intervals for 7 weeks. The remaining rats came to autopsy at various times during the eighth and ninth weeks. Series II consisted of sixteen animals, six of which were placed on diet 16 while ten were given diet 17 but the total intake of these control animals was restricted so that it was essentially equal to that of the phosphorus deficient group. With such paired feeding it was believed that the effect of partial inanition and its influence on the tissues could be satisfactorily judged. These animals came to autopsy from the forty-eighth to the sixty-seventh day.

The animals were killed with ether and autopsies performed immediately. Tissues were placed in Zenker-formol and in formalin alone. After dehydration the tissues were imbedded in paraffin, cut and stained with haematoxylin and eosin. Other special stains were used when indicated. To study the changes in the bones, the distal end of the femur was decalcified in 2% nitric acid and imbedded in paraffin while after decalcification the proximal end of the tibia was imbedded in celloidin. X-rays of the skeleton were taken at varying intervals during the experiments.

Since it seemed inadvisable to examine all the tissues in every animal, an initial study was made of all the organs of animals in series I from the seventh week on. Then those tissues showing changes were studied in the earlier periods and the development of such changes was traced. The same tissues of the rats in series II were likewise examined in

order to determine whether phosphorus deficiency alone or merely restriction of the diet was the factor producing the alterations noted.

*Gross appearance of the animals*

In the animals of series I it was quite apparent after the second week which ones were on diet 16 (low P). These animals were much smaller than the control rats (Day and McCollum, '39). The coats of the deficient animals were rough and unkempt. The skeletal system became greatly rarefied. The bones of the phosphorus low rats were soft and cut with ease. Toward the end of survival, brown encrustations were noted about the nares. There was a great decrease in adipose tissue in the phosphorus deficient animals. The subcutaneous fat and that usually seen in the abdomen and neck was either sparse or entirely lacking. This was noticeable from the second week on. Especially striking was the decrease in perirenal fat and that about the uterine horns. The size of the various organs was decidedly reduced in the deficient animals and seemed to parallel the size of the whole animal. Organ weights were not determined. Jackson and Carleton ('23) have reported such a study in experimental rickets in the rat.

More impressive still were the changes noted in the form of the thorax. The bones became progressively more pliable and because of the respiratory movements the ribs became bowed at the costo-chondral junctions. Thus the capacity of the thorax became less and less due to this sinking in of the lateral walls. This could be quantitatively determined by measuring the amount of water such a thoracic cavity would hold. At 42 days the thorax from a deficient animal had a capacity of 4 cc. while that of a control was 10 cc. Owing to the softening of the ribs there was a marked collapse of the lungs with apparent respiratory difficulty. These findings in the thorax are identical with those observed by Park and Howland ('21) in children with severe rickets.

In contrast to the differences in size between the rats on diets 16 and 17 of series I were the findings in the animals of series II. These latter animals showed little variation as to size. Externally there were few distinguishing features. The bones of the phosphorus low group were more easily bent and cut. The thorax, too, was deformed as was seen in the previous group. However, due to inadequate food intake the thoraces of the control group were small though their capacity was not so reduced as in the deficient animals. There was at autopsy perhaps a little more adipose tissue in the controls but even here the change was not very striking.

## MICROSCOPIC FINDINGS

*Skeletal system.* In the animals of series I changes indicative of rickets were present in the bones after they had been on diet 16 for only 7 days. At this time, there was a broad plate of epiphyseal cartilage due to an increase in number of fully grown cartilage cells. The trabeculae at the cartilage-shaft junction showed osteoid borders and there was a decrease in calcified matrix substance between the rows of cartilage cells. From this time on the ricketic change became progressively marked until the forty-ninth day. At this time due to the slowing of growth the changes were much less conspicuous. X-rays taken at 14 days and at various intervals thereafter seemed to verify the changes that were taking place in the bones. It seems unnecessary to detail the bone changes since they proved to be qualitatively identical with those observed in the classical studies of experimental high calcium-low phosphorus rickets made by Shipley, Park, McCollum and Simmonds ('21) and Sherman and Pappenheimer ('21) in the rat. They also confirm the more recent detailed studies on rickets in the rat by Dodds and Cameron ('34).

In the animals of series II, ricketic changes were also produced in the deficient rats and no such changes were found in the controls on the restricted intake. However, the effect of partial inanition by evidence of a slowing of bone growth was noted.

Even in the presence of moderate vitamin D ricketic changes were produced within 7 days. This is not an unexpected finding, since Querido ('35) has shown that rats on a cereal diet containing 0.35% calcium and 0.12% phosphorus devel-

oped rickets which could not be completely prevented by moderate amounts of vitamin D (in the range employed in our studies). However, up to the present experiments, purified diets extremely low in phosphorus content have not been studied and it has been necessary to greatly restrict the intake of vitamin D in order to produce rickets by the usual methods.

*Teeth.* A few sections of teeth were studied but due to technical difficulties not enough were examined to warrant any conclusions.

*Heart and blood vessels.* No abnormalities were noted in the deficient animals.

*Lungs.* In the first week the lungs of the animals of series I were unaffected. However, from 14 days on, as the difference in size of the control and deficient rats increased, differences in the lungs were also noted. By 21 days, this difference was obvious. The pulmonary alveoli of the control animals were large and fully expanded, while the lungs of the deficient rats had small, incompletely expanded alveoli, some being completely collapsed. This atelectasis was, of course, due to the continuous decrease in the capacity of the thoracic cavity which, as the deficiency advanced, made it progressively harder for the lungs to expand. This interference with respiration and aeration of the blood possibly contributed to the death of the animals. How much this respiratory difficulty contributed to a lessening of activity with resulting decrease in food intake and weight loss is of course mere speculation.

In series II the thoracic differences were present but less striking since the thoracic capacity of the diet restricted controls was much smaller than in the ad libitum controls.

*Thymus.* In animals of series I autopsied in the first and second weeks, no differences could be detected between the control and deficient rats. By the third week, however, the difference was striking enough. The cortical portion of the thymus had decreased in thickness and this was evidently due to a loss of lymphocytes since these cells were fewer and more loosely packed than in the controls. The medulla, too, was a little smaller. From then on there was a progressive shrinkage of the thymus in the deficient animals, both medullary and cortical regions taking part in the change, so that in the eighth and ninth weeks only vestiges of thymic tissue remained. By this time, too, the thymus of animals on the ad libitum control diet had begun to become smaller due probably to age.

In the paired feeding series no appreciable difference could be found between the two groups. It appears, therefore, that the changes which the organs exhibited were due to inanition alone. This has been noted by several observers (Jackson, '25).

*Spleen.* After several weeks on the diet, the spleens of the control animals in series I tended to be larger than those of the phosphorus deficient rats. Microscopic study of the spleens of the latter animals revealed smaller Malpighian bodies and what was taken to be an increase in hemosiderin. However, the Prussian blue reaction revealed that each contained about the same amount of pigment. In our experience the rat's spleen normally contains a good deal of pigment, more than other laboratory animals such as the rabbit and guinea pig. It does not appear therefore, that there was any severe blood destruction in the deficient animals and, as is pointed out below, the kidneys contained no pigment either.

*Liver, gastro-intestinal tract and pancreas.* No differences were noted between the deficient animals and their controls.

*Adrenals.* In series I the adrenal tissue of the deficient animals showed a decided decrease in the thickness of the cortex. All three cortical layers were affected. Frozen sections stained for fat with Scharlach R, showed decidedly less lipid material in the phosphorus low animals. The decrease was mainly in the fascicular and reticular layers. The changes observed seemed identical with those described by Jackson ('19) in chronic inanition in rats. That the changes observed by us also were due to inanition is suggested by the fact that the adrenals of the paired feeding group showed no differences histologically.

*Kidneys.* Except for the differences in the size of the various elements, the kidneys of series I showed no appreciable changes. It is significant that no renal calculi, such as have been recently described by Schneider and Steenbock ('39, '40), were observed in any of our phosphorus deficient rats. The above authors' diet contained 0.04% phosphorus and 0.57% calcium and they state that single calculi were observed as early as the seventh week but large numbers of uroliths were not noted until the twentieth week. Our experiments differ from theirs in that our diet contained less calcium and phosphorus than the one they employed and, moreover, our animals lived at the longest 68 days. Bluish-staining "calcified" material was found in the renal tubules of one of our control

animals, and in our experience, such deposits are not uncommon in the rat, especially in older animals. No iron pigment was found in the tubular epithelium.

*Urinary bladder and ureter.* The mucosa of the urinary passages showed no differences in the deficient and control animals.

*Testes.* In series I the testes of the deficient and control animals autopsied after 7 days were essentially the same. Spermatogonia and spermatocytes with many mitotic figures were seen. There were a few spermatids but no mature spermatozoa. At the end of 14 days the picture in the deficient animals was unchanged while the tubules of the controls were larger and there were large numbers of spermatids. After 21 days, active spermatogenesis was taking place in the controls with numerous spermatozoa, while the deficient rats remained essentially unchanged. After 35 days, however, a few spermatozoa could be found in the tubules of the deficient rats. The difference in tubular size of the two groups was striking from this time on. Spermatogenesis continued in the phosphorus low animals until the forty-ninth day when it practically ceased. There was almost a complete lack of spermatids and only a few spermatozoa. Giant cells were also seen in the lumina of the tubules. In the paired feeding series there was little if any difference between the two groups. Spermatogenesis was taking place in a few tubules but it was not very active. The tubules were small, thus it seemed evident that the differences noted in series I were due to inanition and they resemble the changes which have been so well described by Siperstein ('21).

*Accessory sex organs.* No changes in the prostates, seminal vesicles and coagulating glands were observed in series I during the first 3 weeks. After 28 days, however, there was a definite increase in the size of these organs in the control group. There was evidence of an increased secretion and the epithelial cells became more columnar. In the ensuing weeks there was the same difference in size of these structures. The epithelium of the deficient animals became more columnar yet never appeared as active as that in the controls. By the fifty-sixth day, the epithelium had become less columnar in the deficient animals and there were fewer vacuoles in the individual cells. In the animals of series II differences between the deficient rats and controls were not apparent. The glands and seminal vesicles were small and the epithelium was

low columnar in appearance with few vacuoles. The change noted in series I was again one of inanition since animals of the paired feeding group failed to exhibit such differences. It is interesting to consider the maturation followed by atrophy. That this is possibly due to a decrease in male sex hormone is suggested by observations of Moore and Samuels ('31).

*Ovary.* Female animals in series I coming to autopsy before 28 days showed little difference in the appearance of the sex glands. After this time the ovaries of the controls had more follicles and the follicles looked more mature. The stroma was increased and more compact. No corpora lutea were seen. In contrast the follicles in the deficient animals were smaller and less numerous. The stroma was looser. Follicles undergoing atresia could be found in both. Until the forty-second day, the differences were even more marked since in the control animals large corpora lutea were found while these were absent in the other group. Again, too, the follicles were smaller in the latter animals. From the forty-ninth day on, it was apparent that even in spite of the weight loss occurring in the deficient animals ovulation had taken place in about half of them. However, the corpora lutea were not so large nor so numerous as they were in the controls. In the animals of the paired feeding series autopsied from the forty-ninth to the sixty-seventh day, a difference was noted between the controls and the deficient rats on the low phosphorus diet. The ovaries of the former group were larger and contained mature follicles and corpora lutea. On the other hand the gonads from the latter animals had no corpora lutea and the follicles were smaller and fewer. It is difficult to evaluate these findings. However, in view of the poorer nutrition of the deficient animals as evidenced by the weight (Day and McCollum, '39) it seems not improbable that the factor of inanition was responsible for the differences and that probably the lack of phosphorus did not play an immediate role. This argument is further strengthened by the fact that half the deficient animals of series I revealed evidence of ovulation and the curve of their weight falls between the two groups in the paired feeding experiment.

*Uterus.* The uteri in series I showed very little difference in the first few weeks. There were occasional glands and a few mitotic figures in the controls. At 28 days, however, there was a distinct difference in size between the two. The epithe-

lial cells of the controls were more columnar and showed vacuoles. The glands were more prominent and the connective tissue stroma was denser. In contrast, the epithelium of the deficient animals was lower and showed little evidence of secretory activity. The glands were inconspicuous. After 28 days the differences were marked and the control uteri grew larger and developed in normal fashion while those of the deficient animals remained atrophic. The uteri from the paired feeding animals of series II showed practically no differences so it appears that the changes found in series I were due to inanition.

*Vagina.* In the first 2 weeks, no differences could be noted in the vaginal epithelium of the animals of series I. At the twenty-first day, however, there was definite keratinization of the epithelium of the control rats. The vaginas of the phosphorus deficient animals failed to show this change. During the ensuing weeks the epithelium of the controls preserved the same appearance of estrus while similar changes were not noted in the rats on the deficient diet until the forty-ninth day. At this time keratinization of the epithelium had occurred in about half and could be correlated with the state of maturity of the ovary. In the animals of series II autopsied between the forty-ninth and sixty-seventh days, the vaginal changes were inconstant in the rats on diet 16; one showed multilayered epithelium while two did not. The number of females (five) in this series makes it unwise to draw any conclusions as to the possible role played by a deficiency of phosphorus.

*Salivary glands.* The submaxillary glands were studied in the animals of series I. No changes were detected.

*Thyroid gland.* The follicles of both groups in series I were about the same size and contained the same amount of pink-staining colloid. However, the epithelial cells of the two groups did differ in that the controls had cuboidal cells while the cells in the deficient animals were a little flatter. There was no change in the stroma of the glands. In the thyroids of the paired feeding series, no differences at all could be detected.

*Parathyroid gland.* Serial sections were made through the parathyroids in series I. Studies of the volume by the planimeter method as employed by Pappenheimer ('36) were not carried out. However, an attempt was made to gain an estimate of cell size by drawing a number from each gland with

the camera lucida. These tracings were then measured and the average size of the nuclei computed. As far as could be determined, the nuclear size was an index of the size of the cell. By comparison of the average cell diameters of the control and deficient animals it appeared that the controls tended to be a little larger. The differences were ascribed to a difference in the weights of the animals of the two groups.

This study of the parathyroid glands was approached with great interest because of the close relationship of these structures to phosphorus and calcium metabolism. In previous experiments Luce ('23) had found that when young rats were fed on a calcium deficient diet (0.04%) there was an enlargement of the parathyroids due to hyperplasia of their cells. Pappenheimer ('36) found an increase in volume of the parathyroids in rats whose renal tissue had been greatly reduced in amount. That one of the important functions of the parathyroid glands is to regulate the excretion of phosphorus seems well established. This hypothesis has been further strengthened by recent work of Drake, Albright and Castleman ('37) who found that when rabbits were given large quantities of phosphate, their parathyroids were increased in size due to hyperplasia of the cells. On the basis of this, it was reasonable to expect the glands of our animals to be decreased in size since the renal output of phosphorus was extremely low as shown by metabolic studies (Day and McCollum, '39). It is possible that the presence of vitamin D in the diet prevented any change in the parathyroids since a deficiency of this nutrient may be essential in the production of parathyroid hyperplasia (Wilder and Howell, '36). Then, too, since our experiments lasted only 9 weeks, one must consider the possibility that differences would not manifest themselves in this interval.

*Muscular system.* Sections of striated and smooth muscle from animals of series I showed no lesions.

*Bone-marrow.* Sections of the medullary cavities of the long bones were studied. In these no change in hematopoietic activity could be demonstrated. As was pointed out above, there was an equal amount of iron pigment in the spleens of

the two groups and there was no indication of excess blood destruction as evidenced by absence of iron pigment in the kidneys of the deficient animals. No blood counts or haemoglobin determinations were performed on these animals. In the recent report of Schneider and Steenbock ('39) no evidence of blood destruction could be detected in acute phosphorus deficiency.

*Nervous system.* Several sections of the brain and spinal cord of animals of series I dying from the seventh week on were studied. No lesions were found in the tissues of the phosphorus deficient animals. The inability to use the hind limbs was doubtless not due to an anatomical lesion in the cord but to muscular weakness.

*Hypophysis.* Mann's stain was used to differentiate the various types of cells. No differences could be detected between the size of the cells and the relative numbers of chromophobes and eosinophiles in the animals of series II. The basophiles were scanty in both groups. When those glands were compared with those of the animals of series I there was a detectable atrophy of the cells of the glands of the paired feeding animals. This apparently was similar to the change described by Jackson ('25) and more recently by Werner ('39). The latter author has pointed out that the gonadotrophic function of the hypophysis fails during chronic inanition in the rat and this doubtless accounts for the changes noted in the testes and ovaries of these animals.

*Skin.* Sections of the dorsal integument were made in animals of series I. In the deficient animals the epidermis was not much different from that of the controls with the exception that there was more keratinization of the epidermis of the latter rats. Little difference was noted in the animals on the paired feeding regime. The corium of the normal animals in series I was definitely thicker than that of the phosphorus low rats but in the animals of series II no marked change could be noted; nor were there any changes in the hair follicles of the rats of this group. The follicles of the normal rats of series I were distinctly larger than those of the deficient animals.

## SUMMARY AND CONCLUSIONS

Detailed microscopic studies have been made on tissues from rats restricted to a diet extremely low in phosphorus (0.017%) but adequate in all other respects. Marked skeletal

rarefaction occurs with accompanying rickets. When the factor of inanition is excluded by paired feeding no other definite differences can be detected. The thymus gland and the reproductive system showed the effect of inanition most strongly. The results emphasize the importance of paired feeding in experiments of this nature.

The technical work was performed by Miss Miriam C. Reed.

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## PLATE 1

### EXPLANATION OF FIGURES

Photographs by Mr. Milton Kougl

1 Low power photomicrograph of proximal end of tibia of rat on P-low diet for 14 days. The ricketic change is indicated by the increase in mature cartilage cells and excessive osteoid.

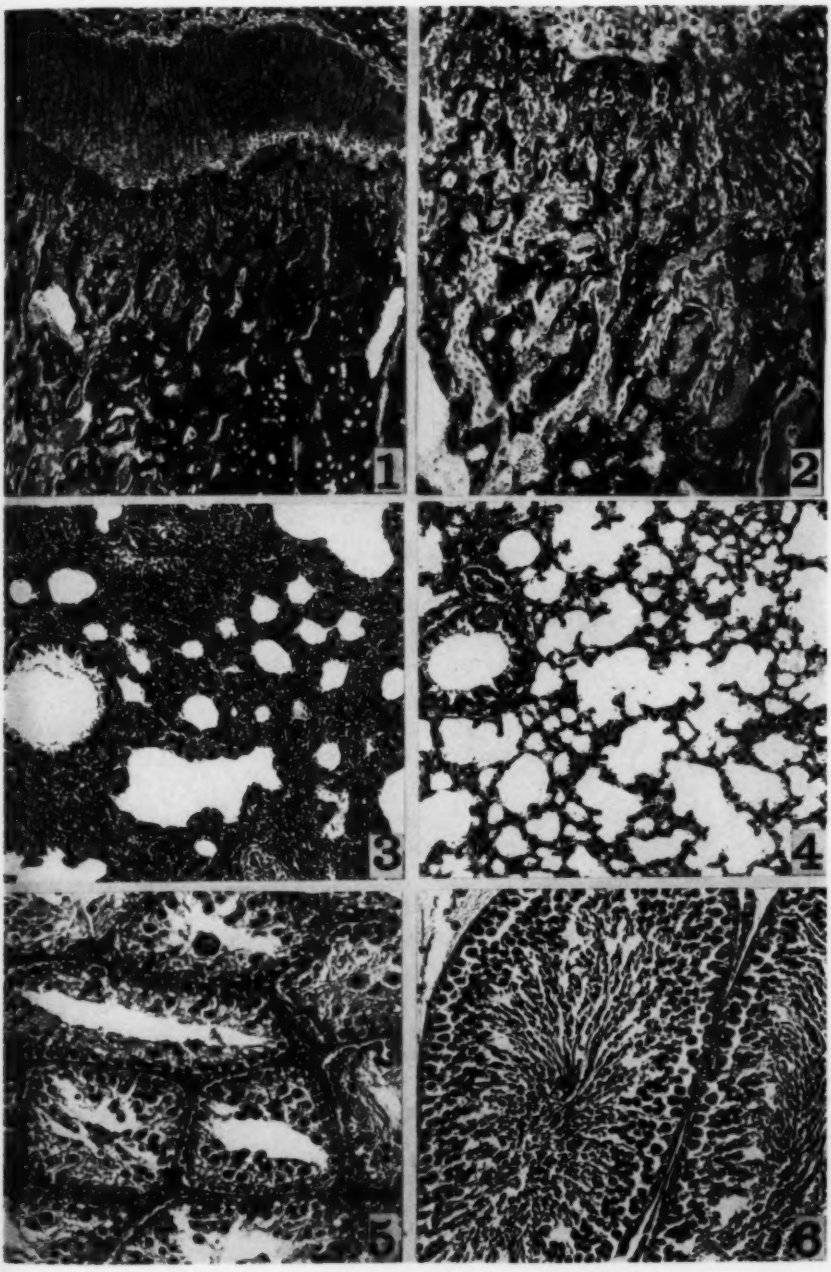
2 Higher magnification of figure 1.

3 Photomicrograph of lung tissue from rat on P-low diet for 49 days. This shows the extreme collapse of most of the alveoli as compared with figure 4.

4 Photomicrograph (same magnification as figure 3) to show normal lung from an ad libitum fed control rat. The alveoli are expanded in contrast to the atelectasis in figure 3.

5 Photomicrograph of testis from animal on P-low diet 53 days. No spermatogenesis is taking place. Note the "giant cells" seen in inanition.

6 Photomicrograph (same magnification as figure 5) of testis from control ad libitum fed animal. The tubules are larger, the cell layers deeper and spermatogenesis is taking place.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